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(54) Title: ANTIBODIES THAT BIND TO AN EPITOPE ON INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR AND USES THEREOF

EICGPGIDIRNDYQQLKRLENCTVIEGYLHILLISKAEDYRSYRFPKLTVITEYLLLFRVAGLESLG
DLFPNLTVIRGWKLFYNYALVIFEMTNLKDIGLYNLRNITRGAIRIEKNADLCYLSTVDWSLILDA
VSNNYIVGNKPPKECGDLCPGTMEEKPMCEKTTINNEYNYRCWTTNRCQKMCPSTCGKRACTE
NNECCHPECLGSCSAPDNDTACVACRHYYYAGVCVPACPPNTYRFEGWRCVDRDFCANILSAE
SSDSEGFVIHDGECMQECPSGFIRNGSQSMYCIPCEGPCPKVCEEEKKTKTIDSVTSAQMLQGCTI
FKGNLLINIRRGNNIASELENFMGLIEVVTGYVKIRHSHALVSLSFLKNLRLILGEEQLEGNYSFYV
LDNQNLQQLWDWDHRNLTIKAGKMYFAFNPKLCVSEIYRMEEVTGTKGRQSKGDINTRNNGER
ASCESDVLHFTSTTTSKNRIIITWHRYRPPDYRDLISFTVYYKEAPFKNVTEYDGQDACGSNSWN
MVDVDLPPNKDVEPGILLHGLKPWTQYAVYVKAVTLTMVENDHIRGAKSEILYIRTNASVPSIPL
DVLSASNSSSQLIVKWNPPSLPNGNLSYYIVRWQRQPQDGYLYRHNYCSKDKIPIRKYADGTIDI
EEVTENPKTEVCGGEKGPCCACPKTEAEKQAEKEEAEYRKVFENFLHNSIFVPRPERKRR

(57) Abstract: The invention relates to the identification of an epitopic region within the extracellular domain of human IGF-1 receptor. Methods of identifying antibodies that specifically bind to the herein disclosed epitopic region together with compositions comprising the antibodies and uses thereof in treating IGF-1 receptor mediated disorders, particularly those that over express IGF-1 receptor are disclosed.

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ANTIBODIES THAT BIND TO AN EPITOPE ON INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR AND USES THEREOF

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the field of immunology, particularly antibodies and uses thereof, particularly methods for treating cancer. More particularly, it provides inhibitory anti-IGF-1R antibodies that specifically bind and compete for a newly discovered discontinuous epitope on the extracellular domain of IGF-1R. The herein disclosed epitope, present at the C and N terminal portions of the extracellular domain of human IGF-1 receptor, together with the methods of the invention may be used to identify additional antibodies useful in antibody-based compositions to treat various IGF-1 receptor mediated disorders and also to extend to the use of immunoconjugates and other therapeutic combinations currently used to treat IGF-1 receptor mediated disorders. The proposed antibodies will find use in treating cell proliferation disorders such as cancer by specifically inhibiting ligand binding and/or down regulating expression of human IGF-1 receptor expressed on various tumors.

2. Description of the Related Art

Recently, the importance of the IGF-1R in cell growth has been confirmed *in vivo* by the finding that mouse embryos with a targeted disruption of the IGF-1R and IGF-2 genes have a size at birth that is only 30% the size of wild type littermates. Liu, *et al.*, Cell, 1993, 75, 59; and Baker, *et al.*, Cell, 1993, 73, 73. 3T3-like cells derived from these mouse embryos devoid of IGF-1Rs (R.sup.- cells) do not grow at all in SFM supplemented by a variety of growth factors, which can sustain the growth of cells derived from wild type littermate embryos (W cells) and other 3T3-cells. Sell, *et al.*, Mol. Cell. Biol., 1994, 14, 3604. R.sup.- cells grow in 10% FBS at a rate that is roughly 40% the rate of W cells, with all phases of the cell cycle being equally elongated. Sell, *et al.*, Mol. Cell. Biol., 1994, 14, 3604. R.sup.- cells are also refractory to transformation by SV40 large T antigen, by an activated ras or a combination of both (Sell, *et al.*, Proc. Natl. Acad. Sci. U.S.A., 1993, 90, 11217; and Sell, *et al.*, Mol. Cell. Biol., 1994, 14, 3604), or by overexpressed growth factor receptors, such as the EGF receptor (Coppola, *et al.*, Mol. Cell. Biol., 1994, 14, 4588), the PDGF β receptor (DeAngelis, *et al.*, J. Cell. Physiol., 1995, 164, 214) and the insulin receptor (Miura, *et al.*, Cancer Res., 1995, 55, 663), all conditions that readily transform cells from wild type littermate embryos or other 3T3-like cells with a physiological number of IGF-1Rs. Conversely, overexpression and/or constitutive activation of IGF-1R in a variety of cell types leads to ligand-dependent growth in SFM and to the establishment of a transformed phenotype. Kaleko, *et al.*, Mol. Cell. Biol., 1990, 10, 464; McCubrey, *et al.*, Blood, 1991, 78, 921; Pietrzkowski, *et al.*, Mol. Cell. Biol., 1992, 12, 3883; Liu, *et al.*, Cell, 1993, 75, 59; Sell, *et al.*, Mol. Cell. Biol., 1994, 14, 3604;

Coppola, *et al.*, Mol. Cell. Biol., 1994, 14, 4588; and Surmacz, *et al.*, Exp. Cell Res., 1995, 218, 370.

The importance of the IGF-1 receptor in the control of cell proliferation is supported by the observation that many cell types in culture are stimulated to grow by IGF-I (Goldring, *et al.*, Crit. Rev. Eukaryot. Gene Expr., 1991, 1, 301; and Baserga, *et al.*, Crit. Rev. Eukaryot. Gene Expr., 1993, 3, 47), and these cell types include human diploid fibroblasts, epithelial cells, smooth muscle cells, T lymphocytes, myeloid cells, chondrocytes, osteoblasts as well as the stem cells of the bone marrow. The important role of IGF-1R in the establishment and maintenance of the transformed phenotype is supported by other findings. Antisense oligonucleotides or antisense expression plasmids against either IGF-2 (Christophori, *et al.*, Nature, 1994, 369, 414; and Rogler, *et al.*, J. Biol. Chem., 1994, 269, 13779), IGF-1 (Trojan, *et al.*, Proc. Natl. Acad. Sci. U.S.A., 1992, 89, 4874; and Trojan, *et al.*, Science, 1993, 259, 94) or the IGF-1R (Sell, *et al.*, Proc. Natl. Acad. Sci. U.S.A., 1993, 90, 11217; Baserga, Cell, 1994, 79, 927; Resnicoff, *et al.*, Cancer Res., 1994, 54, 2218; Resnicoff, *et al.*, Cancer Res., 1994, 54, 4848; and Shapiro, *et al.*, J. Clin. Invest., 1994, 94, 1235), antibodies to the IGF-1R (Arteaga, *et al.*, Breast Canc. Res. Treatm., 1992, 22, 101; and Kalebic, *et al.*, Cancer Res., 1994, 54, 5531), and dominant negative mutants of the IGF-1R (Prager, *et al.*, Proc. Natl. Acad. Sci. U.S.A., 1994, 91, 2181; and Li, *et al.*, J. Biol. Chem., 1994, 269, 32558), can all reverse the transformed phenotype, inhibit tumorigenesis, and induce loss of the metastatic phenotype (Long, *et al.*, Cancer Res., 1995, 54, 1006). An overexpressed IGF-1R has been found to protect tumor cells in vitro from etoposide-induced apoptosis (Sell, *et al.*, Cancer Res., 1995, 55, 303) and, even more dramatically, that a decrease in IGF-1R levels below wild type levels caused massive apoptosis of tumor cells in vivo (Resnicoff, *et al.*, Cancer Res., 1995, 55, 2463).

A major feature of malignant cells is the loss of control over one or more cell cycle elements. These elements range from cell surface receptors to the regulators of transcription and translation (Hunter, Cell, 64:249-70(1991); Cantley *et al.*, Cell, 64:281-302 (1991); Aaronson, Science, 254:1146-51 (1991); Hitwell *et al.*, Science 266:1821-8 (1994); Baserga, Cell, 79:927-30 (1994)). Epidemiologic evidence suggests that the over expression or activation of receptor protein tyrosine kinases is a critical step in several cellular functions including cell proliferation, carcinogenesis, apoptosis, and cell differentiation (Plowman, G. D.; Ullrich, A.; Shawver, L. K.: Receptor Tyrosine Kinases As Targets For Drug Intervention. DN&P (1994) 7: 334 339). Indeed, current data suggests that various human hyper cell proliferative malignancies are characterized by overexpression or up regulation of certain tyrosine kinases. As such, therapeutics that specifically target tyrosine kinase mediated cell signaling will find use in treating various forms of cancers.

One tyrosine kinase that has been implicated in hyper proliferative malignancies is the IGF-1 receptor (IGF-1R). The IGF-1R belongs to the family of tyrosine kinase growth factor receptors (Ullrich, *et al.*, Cell, 1990, 61, 203), and is 70% homologous to the insulin growth factor I receptor (Ullrich, *et al.*, EMBO J., 1986, 5, 503). IGF-1R is widely expressed in many human tissues and cell

types. Under normal physiological conditions, the IGF-1R plays an important role in the regulation of cell growth and differentiation, and in protection from apoptosis.

IGF-1R is synthesized as a single chain proreceptor polypeptide, which is processed by glycosylation, proteolytic cleavage, and covalent bonding to assemble into a mature 460-kDa heterotetramer comprising two alpha-subunits and two beta-subunits. The receptor exists as a heterodimer, with several disulfide bridges. The alpha subunit of this receptor is a 130-135 kDa protein that is entirely extracellular and functions in ligand binding. The ligand-binding domain is located on the extracellular alpha subunit. Approximately one-third of the beta-subunit, w3hioch is a 95-kDa transmembrane protein is extracellular and is connected to the intracellular portion by a single transmembrane domain. The tyrosine kinase catalytic site and the ATP binding site are located on the cytoplasmic portion of the beta-subunit. This activity is implicated in the signaling pathways mediating ligand action which involve autophosphorylation of the beta-subunit and phosphorylation of IGF-IR substrates.

The first step in the transduction pathway leading to IGF-I-stimulated cellular proliferation or differentiation is binding of IGF-I or IGF-II (or insulin at supraphysiological concentrations) to the IGF-I receptor. Binding of the secreted growth factor ligands IGF1 or IGF2 results in activation of the IGF-1 receptor. IGF2, however, binds to the receptor with lower affinity relative to that of IGF1. Ligand binding to the alpha-subunits in the extracellular domain induces changes in receptor conformation and triggers autophosphorylation of the cytoplasmic beta-subunits on specific tyrosine residues, alterations which stimulate catalytic activity and expose and/or create binding sites for downstream signaling proteins.

There is considerable evidence for a role for IGF-I and/or IGF-IR in the maintenance of tumor cells *in vitro* and *in vivo*. IGF-IR levels are elevated in tumors of lung (Kaiser *et al.*, J. Cancer Res. Clin Oncol. 119: 665-668, 1993; Moody *et al.*, Life Sciences 52: 1161-1173, 1993; Macauley *et al.*, Cancer Res., 50: 2511-2517, 1990), breast (Pollak *et al.*, Cancer Lett. 38: 223-230, 1987; Foekens *et al.*, Cancer Res. 49: 7002-7009, 1989; Cullen *et al.*, Cancer Res. 49: 7002-7009, 1990; Arteaga *et al.*, J. Clin. Invest. 84: 1418-1423, 1989), prostate and colon (Remaole-Bennet *et al.*, J. Clin. Endocrinol. Metab. 75: 609-616, 1992; Guo *et al.*, Gastroenterol. 102: 1101-1108, 1992). Deregulated expression of IGF-I in prostate epithelium leads to neoplasia in transgenic mice (DiGiovanni *et al.*, Proc. Natl. Acad. Sci. USA 97: 3455-60, 2000). In addition, IGF-I appears to be an autocrine stimulator of human gliomas (Sandberg-Nordqvist *et al.*, Cancer Res. 53: 2475-2478, 1993), while IGF-I stimulated the growth of fibrosarcomas that overexpressed IGF-IR (Butler *et al.*, Cancer Res. 58: 3021-27, 1998). Further, individuals with "high normal" levels of IGF-I have an increased risk of common cancers compared to individuals with IGF-I levels in the "low normal" range (Rosen *et al.*, Trends Endocrinol. Metab. 10: 136-41, 1999). For a review of the role IGF-I/IGF-I receptor interaction plays in the growth of a variety of human tumors, see Macaulay, Br. J. Cancer, 65: 311-320, 1992.

The cloning of the insulin like growth factor-1 receptor (IGF-1R) (Ullrich *et al.*, EMBO J., 5:2503-12 (1986) has allowed for the definitive demonstration that the IGF-1R signaling cascade plays an important role in tumor proliferation and survival and is implicated in inhibition of tumor apoptosis. Abundant data suggest that the activation of an overexpressed IGF-1R can initiate mitogenesis (Pietrzkowski *et al.*, Cell Growth Diff., 3:199-205 (1992)) and promote ligand-dependent neoplastic transformation (Kaleko *et al.*, Mol. Cell Biol. 10:464-73 (1990)). That IGF-1R plays an important role in the establishment and maintenance of the malignant phenotype is readily apparent from a review of the prior art. See "Insulin-Like Growth Factors: Molecular and Cellular Aspects", LeRoith D, ed., Boca Raton: CRC Press, (1991); Masters *et al.*, Ann. NY Acad. Sci., 692: 89-101 (1993); Humbel, Eur. J. Biochem., 190:445-62 (1990); Sara *et al.*, Physiol. Rev. 70:591-614 (1990); Sussenbach, Prog. Growth Factor Res., 1:33-40 (1989); Bondy *et al.*, Ann. Intern. Med., 120:593-602 (1994)).

It has been shown that cells with disrupted IGF-1R genes will not grow in serum-free medium supplemented with growth factors. In addition these cells cannot be transformed by transfection with SV40T antigen or ras, agents that efficiently transform corresponding wild-type cells (Sell *et al.*, Mol. Cell Biol., 14:3604-12 (1994); Sell *et al.*, Proc. Natl Acad. Sci. USA, 90:11217-21 (1993)). Of equal import is the observation that IGF-1R overexpression is frequently found in various tumors, such as breast, colon, lung, sarcoma, and is often associated with an aggressive phenotype.

Abundant data demonstrate that over-expression of either ligand (IGF) and/or the IGF-1 receptor is a feature of various tumor cell lines and can lead to tumor formation in animal models. Over expression of human IGF-1R has been demonstrated to result in ligand-dependent anchorage-independent growth of NIH 3T3 or Rat-1 fibroblasts and inoculation of these cells has been shown to cause rapid tumor formation in nude mice (Kaleko *et al.*, Mol. Cell. Biol. 10 (1990) 464-473; Prager *et al.*, Proc. Natl. Acad. Sci. USA 91 (1994) 2181-2185).

As well, transgenic mice overexpressing IGF-II specifically in the mammary gland develop mammary adenocarcinoma (Bates *et al.*, Br. J. Cancer 72 (1995) 1189-1193) and transgenic mice overexpressing IGF-II under the control of a more general promoter develop an elevated number and wide spectrum of tumor types (Rogler *et al.*, J. Biol. Chem. 269 (1994) 13779-13784). A representative example among many for human tumors over-expressing IGF-I or IGF-II at very high frequency (>80%) are Small Cell Lung Carcinomas (Quinn *et al.*, J. Biol. Chem. 271 (1996) 11477-11483).

It is now established that a major mode of tumor survival is escape from apoptosis (Fisher, Cell, 78:539-42 (1994)). IGF-1R abrogates progression into apoptosis, both *in vivo* and *in vitro* (Kulik *et al.*, Mol. Cell. Bio., 17: 1595-1606 (1997); Lamm *et al.*, Cancer Res., 58:801(1998)). It has also been shown that a decrease in the level of IGF-1R below wild-type levels causes apoptosis of tumor cells in vivo (Resnicoff *et al.*, Cancer Res., 55:2463-2469 (1995); Resnicoff *et al.*, Cancer Res., 55:3739-3741 (1995)). The ability of IGF-1R disruption to cause apoptosis appears to be diminished in normal, non-tumorgenic cells (Barega, Trends Biotechnol., 14:150-2 (1996)).

The correlation between a reduction of IGF-1R expression and resistance to transformation has been seen in several other systems as well. Prager *et al.* inhibited the growth and transformation of rat-I fibroblasts by introduction of a dominant negative mutant of the IGF-1R (Prager *et al.*, Proc. Natl. Acad. Sci. USA., 91:2181-5 (1994)). Others have used an antisense strategy to demonstrate that interference with the IGF/IGF-1R system is sufficient to reverse the transformed phenotype and to inhibit tumor growth (Trojan *et al.*, Science 259 (1993) 94-97; Kalebic *et al.*, Cancer Res. 54 (1994) 5531-5534; Prager *et al.*, Proc. Natl. Acad., Sci. USA 91 (1994) 2181-2185; Resnicoff *et al.*, Cancer Res. 54 (1994) 2218-2222; Resnicoff *et al.*, Cancer Res. 54 (1994) 4848-4850; Resnicoff *et al.*, Cancer Res. 55 (1995) 2463-2469.

As noted by Burfeind *et al.*, mice injected with rat prostate adenocarcinoma cells (PA-III) transfected with IGF-1R antisense cDNA (729 bp) develop tumors 90% smaller than controls or remained tumor-free after 60 days of observation (Proc. Natl. Acad. Sci. USA 93 (1996) 7263-7268).

Several publications and patent documents are referenced in this application in order to more fully describe the state of the art to which this invention pertains. The disclosure of each of these publications and documents is incorporated by reference herein.

It is clear that elevated levels of the IGF-1 receptor are observed in a variety of human tumor types, and interference with IGF-1 receptor function by antisense strategies, antibodies, or dominant-negative mutants have demonstrated efficacy in reversing the transformed phenotype in a variety of tumor cell lines. For these reasons, the IGF-1 receptor has emerged as a therapeutic target for the treatment of human cancer.

The various *in vitro* and *in viva* strategies, noted above, have provided the necessary proof of principal that inhibition of IGF-1R signaling reverses the transformed phenotype and inhibits tumor cell growth.

However, although IGF-1R inhibitors are promising chemotherapeutic agents, use of these drugs presents a challenge because of the high degree of shared sequence homology between the IGF-1 receptor and the insulin receptor (IR). Overwhelming data further suggest that administration of small molecule IGF-1R inhibitors induces hyperglycemia, presumably through a block of the insulin receptor. See, WO 02/102804. Because of their lack of specificity, current therapies not only target diseased cells but also normal growing cells. Additionally, high doses of cytotoxic agents needed for therapeutic efficacy also destroy normal dividing cells. Radiation and surgical therapies are limited to relatively-localized areas. As well, current therapeutic strategies are limited by degree of deformity and/or disability that patients are willing to tolerate for a modest increase ion survival. As a consequence, conventional therapies are poorly tolerated and become ineffective when the disease is widely metastatic.

Consequently, there is a need in the art to find therapeutic moieties that limit non-specific targeting so that the full potential of these promising new drugs can be realized. Indeed, the

silence in the art of selective IGF-1R inhibitory moieties (antagonists), e.g., those that inhibit IGF-1R and not IR, argues for more efficacious therapeutic moieties.

The present invention provides alternative inhibitory antibodies specific for an epitope on IGF-1R expressing cancer cells and improved methods of treating cancer that overcome the limitations of conventional therapeutic methods as well as offer additional advantages that will be apparent from the detailed description below.

SUMMARY OF THE INVENTION

Provided herein are the results of research detailing the identification of the regions of the primary sequence of the human Insulin-like Growth Factor 1 Receptor (IGF-1R) which are recognized by a humanized monoclonal antibody (mAb) F50035(reference antibody), i.e. the identification of an epitope of the mAb on IGF-1R (a/k/a IGF-1R epitope). The IGF-1 receptor is a receptor tyrosine kinase that shares a second messenger signaling pathway that is substantially similar to HER2 and EGFR. Over expression of IGF-1 receptor has been implicated in numerous hyper proliferative disorders such as cancers of the colon, lung, breast, and prostate. The reference antibody (F50035) has been shown to inhibit IGF-1R autophosphorylation, down-regulate IGF-1 receptor expression, and inhibit ligand-induced cell proliferation. Consequently, this monoclonal antibody is being pursued as a potential target for treating IGF-1 receptor mediated pathologies, especially cancers where this receptor is over expressed relative to normal. The epitope studies conducted by the inventors reveals that the reference antibody binds "conformational" or "discontinuous" epitope on the surface of IGF-1R expressing cells, which comprises non-adjacent stretches of primary sequence. The newly discovered epitope of the invention resides at the N-terminal region of the alpha chain of IGF-1R defined by Gly_{27}-Arg_{41} and the C-terminal region of the alpha chain between amino acids Tyr_{688}-Arg_{707}. The numbers are relative to the sequence information detailed in Ullrich *et al. infra*. The present invention thus relates to antibodies that bind to particular epitopes that are present on cells, such as cancer cells. As a consequence of the epitope data provided herein, the invention thus permits the prediction that additional antibodies can be identified based upon their ability to bind the epitope detailed herein using assays known to a skilled artisan that effectively allow such identification, based, in part, upon the ability of the antibodies to compete with the target antibody to bind the herein detailed epitope on the surface of IGF-1R.

Consequently, in one aspect, the invention provides antibodies that competitively inhibit binding of an antibody to an epitope of the invention as determined by any method known in the art for determining competitive binding, for example, the immunoassays described herein. In preferred embodiments, the antibody competitively inhibits binding to the epitope, e.g., IGF-1R epitope by at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50%.

In its broader aspect, the invention provides a monoclonal antibody (mAb) against human IGF-1R, the antibody recognizing a discontinuous epitope on the surface of IGF-1R. Preferably

the mAb most intensively reacts with a discontinuous epitope located between amino acids at positions 27 through 41 and 688 through 707 wherein the amino acids at positions 27-41 are GYHILLISKAEDYR (SEQ ID NO:1) and said amino acids at positions 688-707 are YRKVFENFLHNSIFVPRPER(SEQ ID NO:2).

The anti-IGF-1R antibodies of the invention , e.g., those that compete for binding to the same epitope as the epitope bound by the reference antibody – F50035 include intact (full length) antibodies as well as antibody fragments, such as single-chain variant fragments, and mimetics thereof (including intrabodies), to the human insulin-like growth factor-1 receptor (IGF-1R). The anti-IGF-1R antibodies of the invention include antibody fusion polypeptides comprising at least the antibody V region sequences fused to a heterologous polypeptide.

A particular advantage of the present invention is that the invention antibodies inhibit ligand binding to IGF-1R as well as a hybrid receptor comprising IGF-1R and IR but not IR. Significantly, the antibodies identified by methods of the invention specifically block ligand binding to IGF-1R and the hybrid receptor at substantially the same epitope as a reference antibody designated F50035 herein. As such, the antibodies of the invention will inhibit cell proliferation and induce tumor regression as effectively as other anti-IGF-1 receptor antibodies, including those already in clinical trials, and yet have improved safety due to their specific blocking properties. As well, the antibodies are expected to down regulate IGF-1R expression and inhibit the attendant tyrosine kinase activity, e.g. autophosphorylation of specific tyrosine residues upon activation of the IGF-1R receptor by a ligand specific thereto. The compositions and methods of the invention will also extend to the use of immunoconjugates and combinations, including prodrugs, using the specific category of antibodies provided.

The invention antibodies, like the reference antibody, internalize upon binding to IGF-1R on a mammalian cell *in vivo*. These antibodies can also target an IGF-1R -expressing tumor cell *in vivo*. As such, the antibodies of the invention may be used as a targeting agent to direct a therapeutic to a specific cell or site within the body. Thus, an advantage attending the antibodies of the invention is that the antibodies can be used to specifically deliver attached therapeutic agents to tumors that over express IGF-1R by virtue of binding to substantially the same epitope as F50035. In the context of immunoconjugates, therefore, the present invention provides agents that have both anti-proliferative and tumor destructive properties within the same molecule.

The present application further describes the methodology for generating candidate ligand-blocking, anti-IGF-1 receptor antibodies and the routine technical aspects of the assays required to identify actual ligand-blocking specific antibodies from the pool of candidates. In light of this invention, therefore, a range of ligand-blocking, anti-IGF-1 receptor antibodies can be made and used in a variety of embodiments, including in the inhibition of hyper cell proliferation and the treatment of cancer and tumors without inhibiting IR.

In a preferred embodiment, the anti-IGF-1R antibody of any of the preceding embodiments is a chimeric or human antibody. In a preferred embodiment, the chimeric antibody is a humanized antibody. The antibodies of the invention include those produced in mammalian or bacterial cells. In another preferred embodiment, the anti-IGF-1R antibodies are fully human antibodies.

The invention antibodies may also be conjugated to a cytotoxic agent. As such, the invention also provides anti-IGF-1R antibodies (that bind the same epitope as F50035) that are conjugated to a cytotoxic agent or to a growth inhibitory agent. The cytotoxic agent can be a toxin, antibiotic, radioactive isotope or nucleolytic enzyme. For example, the toxin may be one of calicheamicin or a maytansinoid such as "DM1".

Thus, a separate embodiment of the invention provides a method of killing an IGF-1R-expressing cancer cell. The proposed method comprises contacting the cancer cell with an anti-IGF-1R antibody of any of the above embodiments, e.g., selecting an antibody that compete for binding to the same epitope as the epitope bound by the reference antibody – F50035, thereby killing the cancer cell. A related embodiment provides for a method of alleviating or treating an IGF-1R-expressing cancer in a mammal, comprising administering a therapeutically effective amount of the anti-IGF-1R antibodies of the invention to the mammal. The proposed method of alleviating the IGF-1R-expressing cancer anticipates administration of the anti-IGF-1R antibody in conjunction with chemotherapy wherein the mammal is also receiving at least one chemotherapeutic agent. For example, the chemotherapeutic agent may be a taxane such as paclitaxel (TAXOL®) or docetaxel, or derivatives and analogs thereof. Preferably, in the preceding two methods, the cancer is a prostate, breast, colon or lung. In a preferred embodiment of these methods, the anti-IGF-1R antibody of the invention is a fully human antibody. Alternatively, it is a chimeric or a humanized antibody.

Certain preferred compositions are therefore compositions comprising at least a first anti-IGF-1 receptor antibody, or antigen-binding fragment thereof, or at least a first purified anti-IGF-1 receptor antibody, or antigen-binding fragment thereof, that binds to substantially the same epitope as the monoclonal antibody F50035; compositions comprising at least a first monoclonal antibody, or antigen-binding fragment thereof, that binds to the IGF-1 receptor at essentially the same epitope as the monoclonal antibody F50035; and compositions comprising at least a first anti-IGF-1 receptor monoclonal antibody, or antigen-binding fragment thereof, that binds to the same epitope as the monoclonal antibody F50035.

Anti-IGF-1 receptor antibodies, or antigen-binding fragments thereof, that bind to substantially the same epitope as the monoclonal antibody F50035 and that specifically inhibits ligand binding to the IGF-1 receptor; and anti-IGF-1 receptor antibodies, or antigen-binding fragments thereof, that bind to substantially the same epitope as the monoclonal antibody F50035 and that inhibit ligand binding to the IGF-1 receptor without significantly inhibiting ligand binding to the IR form other aspects of the invention.

Antibodies with such combinations of properties can be readily identified by one or more or a combination of the receptor competition, ELISA, co-precipitation, and/or functional assays and the F50035-crossreactivity assays, all of which are well known to one skilled in the art. The guidance concerning the quantitative assessment of F50035-like antibodies that consistently significantly reduce ligand binding to IGF-1 receptor and that consistently do not significantly inhibit ligand binding to IR is as described in the art.

The invention also features receptor-specific antibodies which not only prevent ligand binding but prevent receptor activation. Receptor activation (i.e., signaling) may be determined by techniques described herein or otherwise known in the art. For example, receptor activation can be determined by detecting the phosphorylation (e.g., tyrosine or serine/threonine) of the receptor or its substrate by immunoprecipitation followed by western blot analysis, techniques well known to a skilled artisan. Preferably, antibodies identified by the methods of the invention that bind or competitively inhibit binding to the epitopic regions detailed herein inhibit ligand activity or receptor activity by at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50% of the activity in absence of the antibody.

The binding affinity of an antibody to an antigen and the off-rate of an antibody-antigen interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled antigen (e.g., 3H or 125I) with the antibody of interest in the presence of increasing amounts of unlabeled antigen, and the detection of the antibody bound to the labeled antigen. The affinity of the antibody of interest for a particular antigen and the binding off-rates can be determined from the data by scatchard plot analysis. Competition with a second antibody can also be determined using radioimmunoassays. In this case, the antigen is incubated with antibody of interest conjugated to a labeled compound (e.g., 3H or 125I) in the presence of increasing amounts of an unlabeled second antibody.

In another aspect of the invention, binding of a test moiety (binding agent, e.g. candidate antibody) to a three-dimensional motif of the extracellular domain comprising the herein disclosed "conformational" epitope of the IGF-1 receptor is predicted to modulate an activity of the IGF-1 receptor. Consequently, modulating an activity of the IGF-1 receptor reduces or inhibits an activity of the IGF-1 receptor, which is also envisioned by the present invention.

Also provided are methods of screening compounds for the ability to modulate the activity of proteins carrying an IGF-1R epitope, as well as pharmaceutical compositions comprising such agents.

Preferably, the antibodies of the present invention act as antagonists of the receptor protein (IGF-1 receptor) of the present invention by specifically disrupting or blocking the receptor/ligand interactions *in vivo*. IGF-1R antagonists identified by the method of the invention may subsequently be tested in various assays known to one skilled in the art to determine their ability to

modulate (i.e., inhibit) an activity (e.g., down regulating of IGF-1 receptor expression, or tyrosine kinase activity etc) of the IGF-1 receptors.

An exemplary embodiment o f the invention provides a method for screening for an IGF-1 receptor antagonist that inhibits the interaction between IGF- 1R and its binding partner. The binding partner may be IGF-1 or IGF-2.

The method comprises:

a) combining an IGF-1 receptor or cells expressing said receptor and the reference antibody (F50035) or an antigen-binding fragment thereof with a solution containing a candidate antagonist such that the IGF-1receptor and the reference antibody are capable of forming a complex and

b) determining the amount of complex relative to the predetermined level of binding in the absence of said candidate IGF-1 receptor antagonist and therefrom evaluating the ability of said candidate antagonist to inhibit binding of IGF-1R to the reference antibody.

Such a screening assay is preferably performed as an ELISA assay whereby IGF-1R or its binding partner, is bound on a solid phase.

Yet another embodiment of the invention provides a method for the production of a therapeutic agent for the treatment of carcinomas in a patient comprising combining a therapeutically effective amount of an IGF-1 receptor antagonist which inhibits the interaction between IGF-1R and its binding partner in biochemical and/or cellular assays to an extent of at least 50%.

Biochemical assays are preferably ELISA-based assays or homogeneous assays. In the case of the ELISA system antibodies specific for the two binding partners are used for detection of the complexes. In the case of the homogenous assay at least one binding partner is labeled with fluorophores which allows analysis of the complexes. Cellular assays are preferably assays whereby tumor cells or cells transfected with expression constructs of the IGF-1R and the respective binding partners/ proteins are treated with or without drugs and complex formation between the two components is then analyzed using standard cell assays.

A preferred embodiment of the invention is a method for the production of a therapeutic agent for the treatment of carcinomas in a patient comprising combining a pharmaceutically acceptable carrier with a therapeutically effective amount of a compound which inhibits the interaction between IGF-1R and its binding partner/polypeptide, in a cellular assay, whereby in said cellular assay tumor cells or cells transfected with expression constructs of IGF-1 receptor comprising the epitopic regions detailed herein and the binding partner are treated with said test preparation, e.g., sera suspected of containing the candidate antibody, and complex formation between IGF-1R and said respective binding partner is analyzed, and the extent of said complex formation in the case of inhibition does not exceed 50% referred to 100% for complex formation without said candidate antibody containing preparation in said same cellular assay.

Methods of preparing the subject antibodies are also envisioned by the present invention. An exemplary embodiment for preparing anti-IGF-1 receptor antibodies that bind to substantially the

same epitope as the monoclonal antibody F50035 includes a process comprising immunizing an animal with at least a first immunogenic IGF-1R component and selecting from the immunized animal an antibody that substantially cross-reacts with the monoclonal antibody F50035; and anti-IGF-1 receptor antibodies that bind to substantially the same epitope as the monoclonal antibody F50035, prepared by a process comprising immunizing an animal with at least a first immunogenic IGF-1 receptor component and selecting a cross-reactive anti-IGF-1 receptor antibody from the immunized animal by identifying an antibody that substantially reduces the binding of the F50035 antibody to IGF-1 receptor.

In another embodiment, the invention provides a purified and isolated IGF-1R seven transmembrane receptor polypeptide comprising the amino acid sequence set forth in Ullrich *et al.*, or a fragment thereof comprising an epitope specific to the seven transmembrane receptor. Preferably, the epitope is the IGF-1R epitope detailed herein. By "epitope specific to" is meant a portion of the IGF-1R receptor that is recognizable by an antibody that is specific for IGF-1R seven transmembrane receptor, as defined in detail below, e.g., F50035 and additional antibodies that bind the same epitope as F50035.

It will be appreciated that extracellular epitopes are particularly useful for generating and screening for antibodies and other binding compounds that bind to the epitopic region defined herein. Thus, in another preferred embodiment, the invention provides a purified and isolated polypeptide comprising at least one extracellular domain of IGF-1R. By "extracellular domain" is meant the amino terminal extracellular domain or an extracellular loop that spans two transmembrane domains. A purified and isolated polypeptide comprising the N-terminal extracellular domain of IGF-1R is highly preferred that carries the IGF-1R epitope of the invention.

Also preferred is a purified and isolated polypeptide comprising a IGF-1R seven transmembrane receptor fragment selected from the group consisting of the N-terminal extracellular domain of IGF-1R, transmembrane domains of IGF-1R, extracellular loops connecting transmembrane domains of IGF-1R, and intracellular loops connecting transmembrane domains of IGF-1R, and fusions thereof. Such fragments may be continuous portions of the native receptor. However, it will also be appreciated that knowledge of the IGF-1R gene and protein sequences as provided in the art permits recombining of various domains that are not contiguous in the native protein.

The methods of the invention also propose methods of treating IGF-1R mediated pathologies. An exemplary method thus involves the treatment of an individual, preferably a patient, more preferably a mammalian patient and even more preferably a human mammalian patient, having or suspected of having a cell proliferative disorder mediated by over-expression of IGF-1R relative to normal by administering a therapeutically effective amount of an antibody of the invention, which is characterized as binding the same epitope on IGF-1R as the reference antibody. Single-chain variant fragments, or antibody composition comprising a single-chain variant fragment to the individual are also included.

The methods of the invention may also involve the treatment of IGF-1R mediated disorders by expressing anti-IGF-1R antibodies, including single-chain variant fragments, in cells expressing an IGF-1R protein.

In addition, methods and devices are provided for screening samples for the presence of proteins carrying the herein disclosed IGF-1R epitope. The methods may be used, for example, to diagnose a patient as someone who is, or is likely to suffer from an IGF-1R mediated disease or a related disorder.

Nucleic acids encoding the subject antibodies as well as methods for their expression, including in therapeutic treatment protocols, are also provided. Nucleic acids of the invention can be introduced into a host cell using various viral vectors and non-viral delivery techniques for expression of the nucleic acid encoding the antibody in a particular tissue.

As such, in another aspect, the invention provides an isolated nucleic acid encoding any one of the anti-IGF-1R antibodies of the above embodiments, as well as an expression vector comprising the isolated nucleic acid operably linked to an expression regulatory sequence.

Methods of producing the anti-IGF-1R antibodies of the above embodiments, comprising culturing the cells of the above embodiments and recovering the antibody from the cell culture are also provided. Suitable host cells that produce the above-described anti-IGF-1R antibodies are also included. For example, an exemplary embodiment proposes that the antibody producing cells be hybridoma cells. In another embodiment, the cell is a bacterial cell. A host cell comprising the above-described vector is specifically provided.

In yet another embodiment, the invention provides recombinant cells expressing a polypeptide carrying the discontinuous epitope detailed herein, which is bound by F50035. Preferably, the epitope expressed by said cells comprises a conformational epitope comprising a primary sequence of amino acids at positions 27-41 and 688 through 707 as shown in Ullrich *et al.*, wherein the amino acids at positions 27-41 are GYHILLISKAEDYR (SEQ ID NO:1) and said amino acids at positions 688-707 are YRKVFENFLHNSIFVPRPER. (SEQ ID NO: 2).

In another aspect, the invention provides nucleic acid molecules or fragments thereof that encode epitope-bearing portions of the IGF-1 receptor proteins. In particular, such nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising amino acid residues from about 27 through 41 of the terminal region of the alpha chain and amino acid residues from position 688 through 707 of the primary sequence representative of the extra cellular domain of human IGF-1 receptor.

The invention further comprises the use of an antibody according to the invention for the diagnosis of IGF-IR in vitro, preferably by an immunological assay determining the binding between IGF- IR of a sample and the antibody according to the invention.

In another aspect, the present invention provides a composition, e.g. a pharmaceutical composition, containing one or a combination of human monoclonal antibodies, or the antigen-binding portion thereof, of the present invention, formulated together with a pharmaceutically acceptable carrier.

The invention also encompasses a composition comprising any one of the anti-IGF-1R antibodies of the invention, and a carrier. In one embodiment, the antibody in the composition is a human antibody or a humanized form of the monoclonal antibody that compete for binding to the same epitope as the epitope bound by the reference antibody – F50035. In a preferred embodiment, the carrier is a pharmaceutically-acceptable carrier. These compositions can be provided in an article of manufacture or a kit.

In furtherance of the above object, the invention provides an article of manufacture comprising a container and a composition contained therein. The composition for use in the proposed kit comprises an anti-IGF-1R antibody identified by the methods of the invention that specifically compete with the target antibody and bind substantially the same epitope as F50035 and further comprises a package insert indicating that the composition can be used to alleviate or treat an IGF-1R-expressing cell proliferative disorder, such as cancer. Such kits may also comprise IGF-1R, or functional fragments thereof. The antibody and receptor components of the kit may be labeled (e.g., by radioisotopes, fluorescent molecules, chemiluminescent molecules, enzymes or other labels), or may be unlabeled and labeling reagents may be provided. The kits may also contain peripheral reagents such as buffers, stabilizers, etc. Instructions for use can also be provided. The antibodies in the kit may, for example, be useful for identifying other ligands that bind to IGF-1R at substantially the same epitope detailed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 details binding of IGF-1R ECD to bead-immobilized reference antibody.

Figure 2 shows the various peptide fragments resulting from the digestion of the reference antibody/IGF-1R complex with endoproteinase ArgC.

Figure 3 provides a summary of the cleavages observed during the epitope excision study. Since all the identified peptides belong to the IGF-1R α-chain, the rest of the ECD is not shown. The cleaved amino acids are denoted in regular underline, protected amino acids in zigzag underline, and the cysteine residues are denoted in double underline.

Figure 4 summarizes the peptides resulting from the sub-digestions of reference monoclonal antibody/IGF-1R complex when incubated with endoproteinase GluC.

Figure 5 shows the regions of the primary sequence of the alpha chain of the ECD (extracellular domain) of IGF-1R, which form the conformational epitope bound by the reference

antibody. These regions are represented by the boxed area (dashed box/boxes) the dashed boxes indicate the residues which had previously been identified as critical for binding the ligand.

Figure 6 provides a comparison of the epitopic regions of IGF-1R as between a human, rat and mouse.

Figure 7 provides the results of a western blot experiment detailing the use of 7C10 and 13F5 to detect native α2 β2 tetrameric form of recombinant IGf-1R ECD.

Figures 8A and B, together detail the disassociation curves, e.g., disassociation of labeled IGF-1/IGF-1R complexes by the reference antibody.

Figures 9A and 9B detail the western blot data, e.g., recognition of the recombinant extracellular domain of IGf-1R N-deglycosylated (column 1) versus one that is not deglycosylated by monoclonal antibodies A2CHm (A) and 13F5 (B) after SDS-PAGE electrophoresis under mild reducing conditions. See example 4.

Figure 10A & B detail the binding analyses of the reference antibody to the alanine scanning receptor mutants in the regions encompassing the mapped epitopes. Numbers in the x-axis represent the IGF-1R amino acid position that was substituted with Alanine. The y-axis represents the percentage of binding relative to the wild-type (wt) IGF-1R receptor represented in the first column as 100%.

Figure 11 graphically details the binding affinity results measuring the binding affinities of IGf-1R variants, for example - the mini-IGF-1R (a fusion protein comprising amino acid residues 1-458 fused to residues residing at positions 691-707 of the alpha subunit of the IGf-1R extracellular domain) and the truncated IGF-1R moiety (comprising amino acid residues residing at positions 1-458 of the wild type IGF-1R alpha subunit of the ECD) to the reference antibody.

Figure 12A maps the various segments of IGF-1R. Each of Figures 12B –C details the mini-IGF- IR (12B) construct and the truncated IGF-1R construct (12C) in a pV1-JNSA vector with cloning sites labeled – *BgI II* and *Kpn I*.

Figures 12D-F describe the various PCR primers used to for amplify the mini-IGF-1R and the truncated IGF0-1R DNA sequences. The forward primer was used for both constructs.

Figure 12G shows the pV1-JNSA vector map with two cloning sites indicated on the map - *BgI II* and *Kpn I*.

Figure 12H exemplifies an SDS-PAGE gel after staining with Gel-Code blue solution (Pierce). Lane 1, molecular marker; lane 2, IGF1R-α subunit; lane 3, mini-IGF1R moiety; lane 4, truncated IGF-1R (1-458).

Figure 12I - Binding affinities of mini-IGF-1R and truncated IGF-1R with the reference antibody measured with ELISA. The complete extracellular domain of IGF-1R (amino acid 1-902) labeled as Standard in the graph was purchased from R&D Systems.

Figure 13 is a schematic representation of the biosensor capturing assay.

Figure 14 details a Sensorgram of the association and dissociation phase of the reference antibody/analyte complexes for five different concentrations of the respective analytes. See example 8.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. See, e.g. Singleton *et al.*, Dictionary of Microbiology and Molecular Biology 2nd ed., J. Wiley & Sons (New York, N.Y. 1994); Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press (Cold Springs Harbor, N.Y. 1989). For purposes of the present invention, the following terms are defined below.

The present invention relates to antibodies which show benefits for patients in need of antitumor therapy. They provide reduction of tumor growth and a significant prolongation of the time to progression. The antibodies according to the invention have new and inventive properties causing a benefit for a patient suffering from a disease associated with an IGF deregulation, especially a tumor, more particularly cancer.

The invention is based at least in part on the discovery of epitopes or an epitopic region recognized by the IGF-1R specific reference antibody – F50035, described in WO 03/059951 and WO2005058967, each of which is incorporated by reference herein in its entirety. Because F50035 specifically reacts with IGF-1R expressing cells, the invention provides an improved, accurate means to identify which IGF-1R expressing patients will be responsive to a therapeutic IGF-1R specific antibody. The invention, by identifying these novel IGF-1R epitopes, also provides methods of generating an immune response, particularly antibodies, directed against cells bearing the IGF-1R epitope. In a broad aspect, the invention provides anti-IGF-1R antibodies, which bind the same epitope as the reference

antibody, and which internalize upon binding to cell surface IGF-1R on a mammalian cell. In another preferred embodiment, the anti-IGF-1R antibodies destroy or lead to the destruction of tumor cells bearing IGF-1R.

Before the present proteins, nucleotide sequences, and methods are described, it is to be understood that the present invention is not limited to the particular methodologies, protocols, cell lines, vectors, and reagents described, as these may vary. It is also understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not to limit the scope of the present invention.

The singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

All technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention pertains. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of protein chemistry and biochemistry, molecular biology, microbiology and recombinant DNA technology, which are within the skill of the art. Such techniques are explained fully in the literature.

Although any machines, materials, and methods similar or equivalent to those described herein can be used to practice or test the present invention, the preferred machines, materials, and methods are now described. All patents, patent applications, and publications mentioned herein, whether supra or infra, are each incorporated by reference in its entirety.

Before further description of the invention, certain terms employed in the specification, examples and appended claims are, for convenience, collected here.

I. Definitions

As used herein, the terms nucleic acid, polynucleotide and nucleotide are interchangeable and refer to any nucleic acid, whether composed of phosphodiester linkages or modified linkages such as phosphotriester, phosphoramidate, siloxane etc and combinations of such linkages. The terms nucleic acid, polynucleotide and nucleotide also specifically include nucleic acids composed of bases other than the five biologically occurring bases (adenine, guanine, thymine, cytosine and uracil). Without limitation, a nucleic acid may be single-stranded or double stranded. In context of the human IGF-1R, the nucleic acid and deduced amino acid sequence may be found in Ullrich *et al. infra*.

In the molecular biology art, researchers use the terms "percent sequence identity"' "percent sequence similarity" and "percent sequence homology" interchangeably. In this application) these terms shall have the same meaning with respect to nucleic acid sequences only. The term "substantial similarity" or "substantial sequence similarity," when referring to a nucleic acid or fragment thereof, indicates that, when optimally I aligned with appropriate nucleotide insertions or deletions with another nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least about 85%, preferably at least about 90%, and more preferably at least about 95%, 96%, 97%, 98% or 99% of

the nucleotide bases, as measured by any well-known algorithm of sequence identity, such as FASTA, BLAST or Gap, as discussed above.

A polypeptide with similar structure to a IGF-1R polypeptide, a fragment of IGF-1R, extracellular domain of IGF-1R, an anti-IGF-1R antibody or antibody fragment thereof, described herein refers to a polypeptide that has a similar secondary, tertiary or quaternary structure of a IGF-1R polypeptide, a fragment of a IGF-1R polypeptide, an anti- IGF-1R antibody, or antibody fragment thereof, described herein. The structure of a polypeptide can be determined by methods known to those skilled in the art, including but not limited to, X-ray crystallography, nuclear magnetic resonance, and crystallographic electron microscopy.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 75% or 80% sequence identity, preferably at least 90% or 95% sequence identity, even more preferably at least 98% or 99% sequence identity. Preferably, residue positions that are not identical differ by conservative amino acid substitutions. A "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain (R group) with similar chemical properties (e. g., charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. In cases where two or more amino acid sequences differ from each other by conservative substitutions, the percent sequence identity or degree of similarity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art. See, e.g., Pearson, Methods Mol. Biol. 24: 307-31 (1994), herein incorporated by reference. Examples of groups of amino acids that have side chains with similar chemical properties include 1) aliphatic side chains: glycine, alanine, valine, leucine and isoleucine; 2) aliphatic-hydroxyl side chains: serine and threonine; 3) amide-containing side chains: asparagine and glutamine; 4) aromatic side chains: phenylalanine, tyrosine, and tryptophan; 5) basic side chains: lysine, arginine, and histidine; and 6) sulfur- containing side chains are cysteine and methionine. Preferred conservative amino acids substitution groups are: valine leucine- isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamate aspartate, and asparagine-glutamine.

Alternatively, a conservative replacement is any change having a positive value in the PAM250 log-likelihood matrix disclosed in Gonnet *et al.*, Science 256: 1443-45 (1992) incorporated by reference in its entirety. A "moderately conservative" replacement is any change having a nonnegative value in the PAM250 log-likelihood matrix. Sequence similarity for polypeptides, which is also referred to as sequence identity, is typically measured using sequence analysis software. Protein analysis software matches similar sequences using measures of similarity assigned to various substitutions, deletions, and other modifications, including conservative amino acid substitutions. For instance, GCG contains programs such as "Gap" and "Bestfit" which can be used with default parameters to determine sequence homology or sequence identity between closely related polypeptides, such as homologous.

Polypeptides from different species of organisms or between a wild type protein and a mutein thereof. See, e.g., GCG Version 6.1. Polypeptide sequences also can be compared using FASTA using default or recommended parameters, a program in GCG Version 6.1. FASTA (e.g., FASTA2 and FASTA3) provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences (Pearson (1990); Pearson (2000). Another preferred algorithm when comparing a sequence of the invention to a database containing a large number of sequences from different organisms is the computer program BLAST, especially BLASTp or BLASTn, using default parameters. See, e.g., Altschul *et al.*, J. Mol. Biol. 215: 403410(1990); Altschul *et al.*, Nucleic Acids Res. 25:3389-402(1997); herein incorporated by reference. The length of polypeptide sequences compared for homology will generally be at least about l 6 amino acid residues, usually at least about residues, more usually at least about 24 residues, typically at least about 28 residues, and preferably more than about 35 residues. When searching a database containing sequences from a large number of different organisms, it is preferable to compare ammo acid sequences.

"Homology" is defined as the percentage of residues in the amino acid sequence variant that are identical after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. One such computer program is "Align 2", authored by Genentech, Inc., which was filed with user documentation in the United States Copyright Office, Washington, DC 20559, on Dec. 10, 1991.

The similarity between two nucleic acid or protein sequences may be determined by a variety of methods. For example, the similarities may be determined in silico by an algorithm, for example a BLAST algorithm, which is the reference standard used herein. The similarity between two nucleic acid sequences also may be determined by specific hybridization, which means that a nucleic acid will hybridize specifically in a genome to a reference nucleic acid (namely, the EphA2 sequence provided herein or portions thereof). The hybridization conditions for achieving specificity naturally will differ, depending on such factors including, without limitation, the length of sequence overlap of the respective nucleic acids, its (melting temperature) Tm, the specific genome and the assay conditions.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino acid or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide at the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity=number of identical overlapping positions/total number of positions.times.100%). In one embodiment, the two sequences are the same length.

The determination of percent identity between two sequences can be accomplished using

a mathematical algorithm known to those of skill in the art. An example of a mathematical algorithm for comparing two sequences is the algorithm of Karlin and Altschul Proc. Natl. Acad. Sci. USA 87:2264-2268(1990), modified as in Karlin and Altschul Proc. Natl. Acad. Sci. USA 90:5873-5877(1993). The BLASTn and BLASTx programs of Altschul, *et al.* J. Mol. Biol. 215:403-410(1990) have incorporated such an algorithm. BLAST nucleotide searches can be performed with the BLASTn program (score=100, word length=12) to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the BLASTx program (score=50, word length=3) to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* Nucleic Acids Res. 25:3589-3402(1997). Alternatively, PSI-BLAST can be used to perform an iterated search which detects distant relationships between molecules (Id.). When utilizing BLAST, Gapped BLAST, and PSI-BLAST programs, the default parameters of the respective programs (e.g., BLASTx and BLASTn) can be used.

Another example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). The ALIGN program (version 2.0) which is part of the GCG sequence alignment software package has incorporated such an algorithm. Other algorithms for sequence analysis known in the art include ADVANCE and ADAM as described in Torellis and Robotti Comput. Appl. Biosci., 10 :3-5(1994); and FASTA described in Pearson and Lipman Proc. Natl. Acad. Sci. 85:2444-8(1988). Within FASTA, ktup is a control option that sets the sensitivity and speed of the search.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancer.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or a secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

The term amino acid or amino acid residue, as used herein, refers to naturally occurring L amino acids or to D amino acids as described further below with respect to variants. The commonly

used one- and three-letter abbreviations for amino acids are used herein (Bruce Alberts *et al.*, Molecular Biology of the Cell, Garland Publishing, Inc., New York (3d ed. 1994)).

The term "host cell" as used herein refers to the particular subject cell transfected with a nucleic acid molecule and the progeny or potential progeny of such a cell. Progeny may not be identical to the parent cell transfected with the nucleic acid molecule due to mutations or environmental influences that may occur in succeeding generations or integration of the nucleic acid molecule into the host cell genome. The cell that produces an anti-IGF-1R antibody of the invention will include the parent hybridoma cell e.g., the hybridomas that are deposited with the ATCC, as well as bacterial and eukaryotic host cells into which nucleic acid encoding the antibodies have been introduced. Suitable host cells are disclosed below.

Human "IGF-1R" or "insulin-like growth factor 1 receptor" as used herein, refers to a receptor protein whose nucleotide and deduced amino acid sequence sequences are as disclosed in e.g., Ullrich *et al.*, supra. The nucleotide and amino acid sequence of human IGF-1R is provided in FIG. 2 on page 2505 of Ullrich *et al.*, EMBO, 5: 2503-2512 (1986). IGF-1R is a 70 amino acid polypeptide with extensive structural homology to insulin. It exerts it biological effects by binding to a specific receptor on the surface of a target cell. It is a membrane glycoprotein consisting of two alpha subunits and two beta subunits connected by disulfide bonds to form the functional beta-alpha-alpha-beta heterotetrameric receptor complex. Upon binding to the extracellular domain, the native ligand, e.g., IGF-1 stimulates an intracellular, tyrosine specific protein kinase activity which leads to beta subunit autophosphrylation and presumably phosphorylation of cytoplasmic components of an IGF-1-specific signal transfer cascade. The numbering of the amino acids relating to the discontinuous epitope discovered by the present inventors is derived from the numbering detailed in Ullrich *et al.* IGF-1R as used herein includes allelic variants and conservative substitution mutants of the protein which have IGF-1R biological activity. These allelic variants and conservative substitution mutants may be GPI-linked or secreted forms of the protein. Unless indicated, "IGF-1R proteins" and "IGF-1R polypeptides" refer to all fragments and variants of the protein of as shown in Ullrich *et al.*, *supra (*Figure 2*)*, as well as to proteins resulting from the alternate splicing of the genomic DNA sequences encoding proteins having regions of amino acid sequence identity and IGF-1R activity which correspond to the protein of figure 2 as well as IGF-1R allelic variants.

"Antigen" when used herein refers to a substance, such as a particular peptide or protein that can bind to a specific antibody. Preferred antigens include human IGF-1R, particularly the extracellular domain and immunogenic fragments thereof.

The term "antibody" (Ab) as used herein encompasses the various forms of antibodies including but not being limited to whole antibodies, antibody fragments, human antibodies, humanized antibodies and genetically engineered antibodies. These include, for example, monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies), and antibody fragments, as long as they exhibit the desired biological activity.

Antibodies of the invention may also include multimeric forms of antibodies. For example, antibodies of the invention may take the form of antibody dimers, trimers, or higher-order multimers of monomeric immunoglobulin molecules. Multimerization of antibodies may be accomplished through natural aggregation of antibodies or through chemical or recombinant linking techniques known in the art. For example, some percentage of purified antibody preparations (e.g., purified IgG1 molecules) spontaneously form protein aggregates containing antibody homodimers, and other higher-order antibody multimers. Alternatively, antibody homodimers may be formed through chemical linkage techniques known in the art. An exemplary protocol for the formation of antibody homodimers is given in Ghetie *et al.*, Proceedings of the National Academy of Sciences USA (1997) 94:7509-7514, which is hereby incorporated by reference in its entirety. Alternatively, antibodies can be made to multimerize through recombinant DNA techniques. IgM and IgA naturally form antibody multimers through the interaction with the J chain polypeptide. Non-IgA or non-IgM molecules, such as IgG molecules, can be engineered to contain the J chain interaction domain of IgA or IgM, thereby conferring the ability to form higher order multimers on the non-IgA or non-IgM molecules. See, for example, Chintalacharuvu *et al.*, Clinical Immunology 101:21-31 (2001) and Frigerio *et al.*, Plant Physiology 123:1483-94. (2000) both of which are hereby incorporated by reference in their entireties.) ScFv dimers can also be formed through recombinant techniques known in the art; an example of the construction of scFv dimers is given in Goel *et al.*, (2000) Cancer Research 60:6964-6971 which is hereby incorporated by reference in its entirety. Antibody multimers may be purified using any suitable method known in the art, including, but not limited to, size exclusion chromatography.

An "isolated antibody" is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or non-reducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

"Native antibodies" and "native immunoglobulins" define a heterotetrameric glycoprotein composed of two identical light (L) chains and two identical heavy (H) chains[1] The chains

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[1] An IgM antibody consists of 5 of the basic heterotetramer unit along with an additional polypeptide called J chain, and therefore contain 10 antigen binding sites, while secreted IgA antibodies can polymerize to form polyvalent

all exhibit the same general structure of relatively conserved framework regions (FR) joined by three hyper variable regions, also called complementarity determining regions or CDRs. The CDRs from the heavy and the light chains of each pair are aligned by the framework regions, enabling binding to a specific epitope. From N-terminal to C-terminal, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in accordance with the definitions of Kabat Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md. (1987 and 1991)), or Chothia & Lesk J Mol. Biol. 196:901-917 (1987); Chothia *et al.* Nature 342:878-883 (1989). For the structure and properties of the different classes of antibodies, see, e.g., Basic and Clinical Immunology, 8th edition, Daniel P. Stites, Abba I. Terr and Tristram G. Parslow (eds.), Appleton & Lange, Norwalk, Conn., 1994, page 71 and Chapter 6.

The term "variable" refers to the fact that certain segments of the variable domains differ extensively in sequence among antibodies. The V domain mediates antigen binding and define specificity of a particular antibody for its particular antigen. However, the variability is not evenly distributed across the 110-amino acid span of the variable domains. Instead, the V regions consist of relatively invariant stretches called framework regions (FRs) of 15-30 amino acids separated by shorter regions of extreme variability called "hypervariable regions" that are each 9-12 amino acids long. The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit varinous effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

The term "hypervariable region" or "antigen-binding portion of an antibody" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region generally comprises amino acid residues from a "complementarity determining region" or "CDR" (e.g. around about residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the V_L, and around about 1-35 (H1), 50-65 (H2) and 95-102 (H3) in the V_H ; Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (e.g. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the V_L, and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the V_H ; Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)). "Framework" or "FR" regions are those variable domain regions other than

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assemblages comprising 2-5 of the basic 4-chain units along with J chain. In the case of IgGs, the 4-chain unit is generally about 150,000 daltons.

the hypervariable region residues as herein defined. Therefore, the light and heavy chains of an antibody comprise from N-- to C-terminus the domains FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. Especially, CDR3 of the heavy chain is the region which contributes most to antigen binding. CDR and FR regions are determined according to the standard definition of Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop".

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier "monoclonal" is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies useful in the present invention may be prepared by the hybridoma methodology first described by Kohler *et al.*, Nature, 256:495 (1975), or may be made using recombinant DNA methods in bacterial, eukaryotic animal or plant cells (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, Nature, 352:624-628 (1991) and Marks *et al.*, J. Mol. Biol., 222:581-597 (1991), for example.

The monoclonal antibodies herein include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity . Chimeric antibodies comprising a murine variable region and a human constant region are especially preferred. Chimeric antibodies are usually prepared by recombinant DNA techniques. Such murine/human chimeric antibodies are the product of expressed immunoglobulin genes comprising DNA segments encoding murine immunoglobulin variable regions and DNA segments encoding human immunoglobulin constant regions. Such "chimeric" antibodies are also referred to as "class- switched antibodies." Methods for producing chimeric antibodies involve conventional recombinant DNA and gene transfection techniques now well known in the art. See, e.g., Morrison, S. L., *et al.*, Proc. Natl. Acad Sci. USA 81 (1984) 6851-6855; U.S. Pat. Nos. 5,202,238 and 5,204,244.

An "antibody fragment" comprises a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab',

$F(ab')_2$, and Fv fragments; diabodies; linear antibodies (see U.S. Pat. No. 5,641,870, Example 2; Zapata *et al.*, Protein Eng. 8(10): 1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, and a residual "Fc" fragment, a designation reflecting the ability to crystallize readily. The Fab fragment consists of an entire L chain along with the variable region domain of the H chain (V_H), and the first constant domain of one heavy chain (C_H 1). Each Fab fragment is monovalent with respect to antigen binding, i.e., it has a single antigen-binding site. On the other hand, pepsin treatment of an antibody yields a single large $F(ab')_2$ fragment which roughly corresponds to two disulfide linked Fab fragments having divalent antigen-binding activity and is still capable of cross-linking antigen. Fab' fragments differ from Fab fragments by having additional few residues at the carboxy terminus of the CH 1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The Fc fragment comprises the carboxy-terminal portions of both H chains held together by disulfides. The effector functions of antibodies are determined by sequences in the Fc region, which region is also the part recognized by Fc receptors (FcR) found on certain types of cells. See below.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This fragment consists of a dimer of one heavy- and one light-chain variable region domain in tight, non-covalent association. From the folding of these two domains emanate six hypervariable loops (3 loops each from the H and L chain) that contribute the amino acid residues for antigen binding and confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

"Single-chain Fv" also abbreviated as "sFv" or "scFv" are antibody fragments that comprise the V_H and V_L antibody domains connected into a single polypeptide chain. Preferably, the sFv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv, see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994); Borrebaeck 1995, infra.

The term "diabodies" refers to small antibody fragments prepared by constructing sFv fragments (see preceding paragraph) with short linkers (about 5-10 residues) between the V_H and V_L domains such that inter-chain but not intra-chain pairing of the V domains is achieved, resulting in a bivalent fragment, i.e., fragment having two antigen-binding sites. Bispecific diabodies are heterodimers of two "crossover" sFv fragments in which the V_H and V_L domains of the two antibodies are present on different polypeptide chains. Diabodies are described more fully in, for example, EP 404,097; WO

93/11161; and Hollinger *et al.*, Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993).

A "native sequence" polypeptide is one which has the same amino acid sequence as a polypeptide (e.g., antibody) derived from nature. Such native sequence polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. Thus, a native sequence polypeptide can have the amino acid sequence of a naturally occurring human polypeptide, murine polypeptide, or polypeptide from any other mammalian species.

The term "amino acid sequence variant" refers to a polypeptide that has amino acid sequences that differ to some extent from a native sequence polypeptide. Ordinarily, amino acid sequence variants of IGF-1R will possess at least about 70% homology with the native sequence IGF-1R, preferably, at least about 80%, more preferably at least about 85%, even more preferably at least about 90% homology, and most preferably at least 95%. The amino acid sequence variants can possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence of the native amino acid sequence.

The phrase "functional fragment or analog" of an antibody is a compound having qualitative biological activity in common with a full-length antibody. For example, a functional fragment or biologically equivalent or immunologically equivalent fragment of IGF-1R is one, which exhibits properties substantially similar to the antibody from which it is derived, e.g., anti-IGF-1R antibody.

An antibody having a "biological characteristic" of a designated antibody, such as the control monoclonal antibody F50035, is one which possesses one or more of the biological characteristics of that antibody which distinguish it from other antibodies that bind to the same antigen, IGF-1R. For example, an antibody with a biological characteristic of F50035 will bind the same epitope as that bound by F50035 (e.g. which competes for binding or blocks binding of monoclonal antibody F50035 to IGF-1R), be able to target a IGF-1R expressing tumor cell in vivo and will internalize upon binding to IGF-1R on a mammalian cell *in vivo*.

"Invention antibody" "antibody of the invention" "subject antibody" "anti-IGF-1R antibody" etc. refers to antibodies identified using the methods detailed herein. Such antibodies, by definition, recognize and bind to the IGF-1R epitope detailed herein. Preferably, the invention antibodies are identified in known assay methods and bind the same epitope as the reference antibody- F50035. The internalizing anti-IGF-1R antibodies of the invention can be in the different forms encompassed by the definition of "antibody" herein. Thus, the invention antibodies include full length or intact antibody, antibody fragments, native sequence antibody or amino acid variants, humanized, chimeric or fusion antibodies, immunoconjugates, and functional fragments thereof. The antibodies can be modified in the Fc region to provide desired effector functions. As discussed in more detail in the sections below, with the appropriate Fc regions, the naked antibody bound on the cell surface can induce cytotoxicity, e.g., via antibody-dependent cellular cytotoxicity (ADCC) or by recruiting complement in complement dependent cytotoxicity, or some other mechanism. Alternatively, where it is desirable to eliminate or reduce effector function, so as to minimize side effects or therapeutic complications, certain other Fc regions

may be used.

Whether an anti-IGF-1R antibody of the invention internalizes upon binding IGF-1R on a mammalian cell can be determined by various assays known to one skilled in the art. For example, to test internalization *in vivo*, the test antibody is labeled and introduced into an animal known to have IGF-1R expressed on the surface of certain target cells. The antibody can be radiolabeled or labeled with fluorescent or gold particles, for instance. Animals suitable for this assay include a mammal such as a NCR nude mouse that contains a human IGF-1R-expressing tumor transplant or xenograft, or a mouse into which cells transfected with human IGF-1R have been introduced, or a transgenic mouse expressing the human IGF-1R transgene. Appropriate controls include animals that did not receive the test antibody or that received an unrelated antibody, and animals that received an antibody to another antigen on the cells of interest, which antibody is known to be internalized upon binding to the antigen (e.g., HERCEPTIN which binds to Her2 expressed on the human breast tumor cell line, MCF-7). The antibody can be administered to the animal, e.g., by intravenous injection. At suitable time intervals, tissue sections of the animal can be prepared using known methods or as described in the experimental examples below, and analyzed by light microscopy or electron microscopy, for internalization as well as the location of the internalized antibody in the cell.

For internalization *in vitro*, the cells can be incubated in tissue culture dishes in the presence or absence of the relevant antibodies added to the culture media and processed for microscopic analysis at desired time points. The presence of an internalized, labeled antibody in the cells can be directly visualized by microscopy or by autoradiography if radiolabeled antibody is used. Alternatively, in a quantitative biochemical assay, a population of cells comprising IGF-1R-expressing cells are contacted in vitro or in vivo with a radiolabeled test antibody and the cells (if contacted in vivo, cells are then isolated after a suitable amount of time) are treated with a protease or subjected to an acid wash to remove uninternalized antibody on the cell surface. The cells are ground up and the amount of protease resistant, radioactive counts per minute (cpm) associated with each batch of cells is measured by passing the homogenate through a scintillation counter. Based on the known specific activity of the radiolabeled antibody, the number of antibody molecules internalized per cell can be deduced from the scintillation counts of the ground-up cells. Cells are "contacted" with antibody *in vitro* preferably in solution form such as by adding the cells to the cell culture media in the culture dish or flask and mixing the antibody well with the media to ensure uniform exposure of the cells to the antibody. Instead of adding to the culture media, the cells can be contacted with the test antibody in an isotonic solution such as PBS in a test tube for the desired time period. In vivo, the cells are contacted with antibody by any suitable method of administering the test antibody such as the methods of administration described below when administered to a patient.

The faster the rate of internalization of the antibody upon binding to the IGF-1R expressing cell *in vivo*, the faster the desired killing or growth inhibitory effect on the target IGF-1R-expressing cell can be achieved, e.g., by a cytotoxic immunoconjugate. Preferably, the kinetics of

internalization of the anti-IGF-1R antibodies are such that they favor rapid killing of the IGF-1R-expressing target cell. Therefore, it is desirable that the anti-IGF-1R antibody exhibit a rapid rate of internalization preferably, within 24 hours from administration of the antibody in vivo, more preferably within about 12 hours, even more preferably within about 30 minutes to 1 hour, and most preferably, within about 30 minutes. As such, the present invention provides anti-IGF-1R antibodies that internalize as fast as about 15 minutes from the time of introducing the anti-IGF-1R antibody *in vivo*. The antibody will preferably be internalized into the cell within a few hours upon binding to IGF-1R on the cell surface, preferably within 1 hour, even more preferably within 15-30 minutes.

"Biological property" or "biological activity" is a biological function caused by an antibody or other compound of the invention. With regard to the IGF-1R specific antibodies, biological activity refers, in part, to the ability to specifically bind to IGF-1R or a portion or fragment thereof that carries the IGF-1R epitope. Specifically, it refers to antibodies that compete with binding to same epitope as the reference antibody. Other preferred biological activities include preventing binding of a native ligand to IGF-1R , tyrosine phosphorylation et c.

"Humanized" forms of non-human (e.g., rodent) antibodies are chimeric antibodies that contain minimal sequence derived from the non-human antibody. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or non-human primate having the desired antibody specificity, affinity, and capability. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al.*, Nature 321:522-525 (1986); Riechmann *et al.*, Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992).

In one aspect, the invention provides a chimeric humanized antibody, wherein a murine CDR is grafted into the framework region of a human antibody to prepare the "humanized antibody." See, e.g., Riechmann, L., *et al.*, Nature 332 (1988) 323-327; and Neuberger, M. S., *et al.*, Nature 314 (1985) 268-270. Particularly preferred CDRs correspond to those representing sequences recognizing the epitopes recognized by F50035.

As used herein, an anti-IGF-1R antibody that "internalizes" is one that is taken up by (i.e., enters) the cell upon binding to IGF-1R on a mammalian cell (i.e. cell surface IGF-1R). The internalizing antibody includes antibody fragments, human or humanized antibody and antibody

conjugate. For therapeutic applications, internalization in vivo is contemplated. The number of antibody molecules internalized will be sufficient or adequate to kill a IGF-1R-expressing cell, especially a IGF-1R-expressing cancer cell. Depending on the potency of the antibody or antibody conjugate, in some instances, the uptake of a single antibody molecule into the cell is sufficient to kill the target cell to which the antibody binds. For example, certain toxins are highly potent in killing such that internalization of one molecule of the toxin conjugate to the antibody is sufficient to kill the tumor cell.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The constant regions of the antibody are constant regions of human IgG1 type. Such regions can be allotypic and are described by, e.g., Johnson, G., and Wu, T. T., Nucleic Acids Res. 28 (2000) 214-218 and the databases referenced therein and are useful as long as the properties of induction of ADCC and preferably CDC according to the invention are retained.

The term "antagonist" when used to refer to an antibody is used in the broadest sense, and includes an antibody that partially or fully blocks, inhibits, or neutralizes a biological activity of a native IGF-1R protein disclosed herein. Methods for identifying antagonists of a IGF-1R polypeptide may comprise contacting a IGF-1R polypeptide or a cell expressing IGF-1R on the cell surface, with a candidate antagonist antibody and measuring a detectable change in one or more biological activities normally associated with the IGF-1R polypeptide.

An "antibody that inhibits the growth of tumor cells expressing IGF-1R" or a "growth inhibitory" antibody is one which binds to and results in measurable growth inhibition of cancer cells expressing or overexpressing IGF-1R. Preferred growth inhibitory anti-IGF-1R antibodies inhibit growth of IGF-1R-expressing tumor cells (e.g., prostate cancer cells) by greater than 20%, preferably from about 20% to about 50%, and even more preferably, by greater than 50% (e.g. from about 50% to about 100%) as compared to the appropriate control, the control typically being tumor cells not treated with the antibody being tested. Growth inhibition can be measured at an antibody concentration of about 0.1 to 30 .mu.g/ml or about 0.5 nM to 200 nM in cell culture, where the growth inhibition is determined 1-10 days after exposure of the tumor cells to the antibody. Growth inhibition of tumor cells in vivo can be determined in various ways such as is described in the Experimental Examples section below. The antibody is growth inhibitory *in vivo* if administration of the anti-IGF-1R antibody at about 1 .mu.g/kg to about 100 mg/kg body weight results in reduction in tumor size or tumor cell proliferation within about 5 days to 3 months from the first administration of the antibody, preferably within about 5 to 30 days.

Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic cells and are absolutely required for such killing. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. No. 5,500,362 or U.S. Pat. No. 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, e.g., in a animal model such as that disclosed in Clynes *et al.* PNAS (USA) 95:652-656 (1998). The method for assessing ADCC proposes treating a preparation of IGF-IR expressing cells with an antibody according to the invention in the presence of effector cells such as freshly isolated PBMC or purified effector cells from buffy coats, like monocytes or NK cells. ADCC is found if the antibody induces at a concentration of 100 nM the lysis (cell death) of 20% or more of the tumor cells after 24 hours. The assay is performed preferably with 51Cr labeled tumor cells and measurement of specifically released 51Cr. Controls include the incubation of the tumor target cells with effector cells but without the antibody.

"Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see review M. in Daeron, Annu. Rev. Immunol. 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, Annu. Rev. Immunol. 9:457-92 (1991); Capel *et al.*, Immunomethods 4:25-34 (1994); de Haas *et al.*, J. Lab. Clin. Med. 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al.*, J. Immunol. 117:587 (1976) and Kim *et al.*, J. Immunol. 24:249 (1994)).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK

cells being preferred. The effector cells may be isolated from a native source, e.g. from blood.

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass) which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro *et al.*, J. Immunol. Methods 202:163 (1996), may be performed.

The term "recombinant human antibody", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from a host cell such as a NSO or CHO cell or from an animal (e.g. a mouse) that is transgenic for human immunoglobulin genes or antibodies expressed using a recombinant expression vector transfected into a host cell. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences in a rearranged form. The recombinant human antibodies according to the invention may also be subjected to *in vivo* somatic hypermutation. Thus, the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire in vivo.

The terms "specific binding" or "specifically binding", as used herein, in reference to the interaction of an antibody and a protein or peptide, mean that the interaction is dependent upon the presence of a particular structure (i.e., the antigenic determinant or epitope) on the protein; in other words, the antibody is recognizing and binding to a specific protein structure rather than to proteins in general. For example, if an antibody is specific for epitope "A", the presence of a protein containing epitope A (or free, unlabeled A) in a reaction containing labeled "A" and the antibody will reduce the amount of labeled A bound to the antibody. Unless otherwise defined in the specification, specific binding or immunospecific binding by an anti-IGF-1R antibody means that the anti-IGF-1R antibody of the invention binds IGF-1R or the hybrid receptor described *supra*, but does not significantly bind to IR, provided that the receptor carries the IGF-1R epitope..

It is well known that the portion of a protein bound by an antibody is known as the epitope. The term "epitope" is used to refer to binding sites for (monoclonal or polyclonal) antibodies on protein antigens. An epitope may either be linear (i.e., comprised of sequential amino acids residues in a protein sequences) or conformational (i.e., comprised of one or more amino acid residues that are not contiguous in the primary structure of the protein but that are brought together by the secondary, tertiary or quaternary structure of a protein). Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

An antibody binds "essentially the same epitope" as a reference antibody, when the two antibodies recognize identical or sterically overlapping epitopes. There are many methods known in the art for mapping and characterizing the location of epitopes on proteins, including solving the crystal structure of an antibody-antigen complex, competition assays, gene fragment expression assays, and

synthetic peptide-based assays, as described, for example, in Chapter 11 of Harlow and Lane, Using Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1999. The most widely used and rapid methods for determining whether two epitopes bind to identical or sterically overlapping epitopes are competition assays, which can be configured in all number of different formats, using either labeled antigen or labeled antibody. Usually, the antigen is immobilized on a 96-well plate, and the ability of unlabeled antibodies to block the binding of labeled antibodies is measured using radioactive or enzyme labels.

According to the gene fragment expression assays, the open reading frame encoding the protein is fragmented either randomly or by specific genetic constructions and the reactivity of the expressed fragments of the protein with the antibody to be tested is determined. The gene fragments may, for example, be produced by PCR and then transcribed and translated into protein in vitro, in the presence of radioactive amino acids. The binding of the antibody to the radioactively labeled protein fragments is then determined by immunoprecipitation and gel electrophoresis. Certain epitopes can also be identified by using large libraries of random peptide sequences displayed on the surface of phage particles (phage libraries).

Alternatively, a defined library of overlapping peptide fragments can be tested for binding to the test antibody in simple binding assays. The latter approach is suitable to define linear epitopes of about 5 to 15 amino acids. An antibody is said to specifically bind an antigen when the dissociation constant is <1 nM, preferably <100 nM, preferably <10 nM, and most preferably <1 nM.

It will be appreciated that extracellular epitopes are particularly useful for generating and screening for antibodies and other binding compounds that bind to receptors such as IGF-1R. Thus, in another preferred embodiment, the invention provides a purified and isolated polypeptide comprising at least one extracellular domain of IGF-1R. By "extracellular domain" is meant the amino terminal extracellular domain comprising the extracellular sequence of both the alpha and the beta subunits. The IGF-1R is a disulfide-bridged dimer. Each monomer is formed of an alpha and a beta subunit. The TM domain is in the beta subunit. The epitope of the reference antibody (F-50035) is all in the alpha subunit (which does not include a TM domain). A purified and isolated polypeptide comprising the N-terminal extracellular domain of the alpha subunit of IGF-1R is highly preferred, it being understood that the extracellular domain of the alpha subunit carry the IGF-1R conformational epitope detailed herein. Also preferred is a purified and isolated polypeptide comprising a IGF-1R transmembrane receptor fragment selected from the group consisting of the N-terminal extracellular domain of IGF-1R, transmembrane domains of IGF-1R, extracellular sequence of the alpha chain of IGF-1R, extracellular sequence of the beta chain of IGF-1R, extracellular sequence of the alpha and beta chains, intracellular sequence of the beta chain of IGF-1R, and fusions thereof so long as the polypeptide carries the conformational epitope detailed herein. All these polypeptides can be present as monomers, or as dimers, where the monomers are linked with disulfide bonds or linkers of any nature known in the art.

Given that IGF-1R -specific antibodies bind to epitopes of IGF-1R, an antibody that specifically binds IGF-1R may or may not bind fragments of IGF-1R and/or variants of IGF-1R (e.g., proteins that are at least 90% identical to IGF-1R) depending on the presence or absence of the epitope bound by a given IGF-1R-specific antibody in the IGF-1R fragment or variant. Likewise, IGF-1R-specific antibodies of the invention may bind species orthologues of IGF-1R (including fragments thereof) depending on the presence or absence of the epitope recognized by the antibody in the orthologue. Additionally, IGF-1R-specific antibodies of the invention may bind modified forms of IGF-1R, for example, IGF-1R fusion proteins, comprising the IGF-1R epitope disclosed herein. In such a case when antibodies of the invention bind IGF-1R fusion proteins, the antibody must make binding contact with the IGF-1R moiety of the fusion protein in order for the binding to be specific. Antibodies that specifically bind to IGF-1R can be identified, for example, by immunoassays or other techniques known to those of skill in the art, e.g., the immunoassays described in the Examples below. The term "binding to IGF-IR" as used herein means the binding of the antibody to IGF-IR in an *in vitro* assay, preferably in a binding assay in which the antibody is bound to a surface and binding of IGF-IR is measured by Surface Plasmon Resonance (SPR). Binding means a binding affinity (K D) of 10-8 M or less, preferably 10-11 to 10-8 M.

The term "surface plasmon resonance", as used herein, refers to an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, N.J.). For further descriptions, see Jonsson, U., *et al.* Ann. Biol. Clin. 51:19-26 (1993); Jonsson, U., *et al.* Biotechniques 11:620-627 (1991); Johnsson, B., *et al.* J. Mol. Recognit. 8:125-131 (1995); and Johnsson, B., *et al.* Anal. Biochem. 198:268-277 (1991).

The term Koff', refers to the off rate constant for dissociation of an antibody from the antibody/antigen complex.

The term "Kd" refers to the dissociation constant of a particular antibody antigen interaction.

Binding to IGF-IR can be investigated by a BIAcore assay (Pharmacia Biosensor AB, Uppsala, Sweden). The affinity of the binding is defined by the terms ka (rate constant for the association of the antibody from the antibody/antigen complex, kd (dissociation constant), and K D (kd/ka). The antibodies according to the invention preferably show a KD of 10-9 M or less.

The term "inhibiting the binding of IGF-I and IGF-II to IGF-IR" as used herein refers to inhibiting the binding of I 125- labeled IGF-I or IGF-II to IGF-IR presented on the surface of appropriate host cells, e.g., HT29 tumor cells in an *in vitro* assay. The inhibition is measured as IC 50 in an assay for binding of IGF-I/IGF-II to IGF- IR on tumor cells. Generally, in such an assay, the amount of radiolabeled IGF-I or IGF-II or IGF-IR binding fragments thereof bound to the IGF-IR provided at the surface of said tumor cells (e.g. HT29) is measured without and with increasing concentrations of the antibody. The IC50 values of the antibodies according to the invention for the binding of IGF-I and IGF-

II to IGF-IR are no more than 10 nM and the ratio of the IC50 values for binding of IGF-I/IGF- II to IGF-IR is about 1:3 to 3:1.

The term "complete inhibition of IGF-I mediated signal transduction" refers to the inhibition of IGF-I-mediated phosphorylation of IGF-IR. For such an assay, IGF-IR expressing cells, preferably H322M cells, are stimulated with IGF-I and treated with an antibody according to the invention (an antibody concentration of 10 nM or lower (IC 50) is useful). Subsequently, an SDS PAGE is performed and phosphorylation of IGF-I is measured by Western blotting analysis with an antibody specific for phosphorylated tyrosine. Complete inhibition of the signal transduction is found if on the Western blot visibly no band can be detected which refers to phosphorylated IGF-IR.

The antibodies according to the invention show a binding to the same epitope of IGF-IR as antibody F50035 or are inhibited in binding to IGF-IR due to steric hindrance of binding by the reference antibody, e.g., F50035. Binding inhibition can be detected by an SPR assay using immobilized antibody 1A and IGF-IR at a concentration of 20-50 nM and the antibody to be detected at a concentration of 100 nM. A signal reduction of 50% or more shows that the antibody competes with antibody 1A. Such an assay can be performed in the same manner by using antibody 8 or 23 as immobilized antibodies.

The antibodies according to the invention include, in addition, such antibodies having "conservative sequence modifications", nucleotide and amino acid sequence modifications which do not affect or alter the above- mentioned characteristics of the antibody according to the invention. Modifications can be introduced by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions include ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g. glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan) , nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a human anti-IGF-IR antibody can be preferably replaced with another amino acid residue from the same side chain family. Amino acid substitutions can be performed by mutagenesis based upon molecular modeling as described by Riechmann, L., *et al.*, Nature 332 (1988) 323-327 and Queen, C., *et al.*, Proc. Natl. Acad. Sci. USA 86 (1989) 10029-10033.

In one aspect, the antibodies according to the invention may include additional characteristics selected from the group consisting of such parameters as (i) binding parameters ka, kd and K D, (ii) binding to the same epitope to which antibody F50035 binds, the IC_{50} values for inhibition of binding of IGF-I and IGF- II to IGF-IR on tumor cells, and the IC_{50} values for inhibition of phosphorylation of IGF-IR upon stimulation of IGF-I in tumor cells. Inhibition of phosphorylation of

IGF- IR leads to the inhibition of phosphorylation of downstream elements such as PkB, the down-regulation of IGF-IR in tumor cells, and the influence on the three-dimensional growth of tumor cells in vitro. The antibodies may further be characterized by their pharmacokinetic and pharmacodynamic values, and the cross-reactivity for other species.

An "IGF-1R-expressing cell" is a cell which expresses endogenous or transfected IGF-1R on the cell surface. An "IGF-1R-expressing cancer" is a cancer comprising cells that have IGF-1R protein present on the cell surface. An "IGF-1R-expressing cancer" produces sufficient levels of IGF-1R on the surface of cells thereof, such that an anti-IGF-1R antibody can bind thereto and have a therapeutic effect with respect to the cancer. The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth or hyper cell proliferation. Examples of cancer include, but are not limited to, as cancers of the colon, lung, breast and prostate.

A "hyper cell proliferative cancer" or a "cell proliferative disorder" defines a cancer which "overexpresses" IGF-1R in that it has significantly higher levels of IGF-1R at the cell surface thereof, compared to a noncancerous cell of the same tissue type. Such overexpression may be caused by gene amplification or by increased transcription or translation. IGF-1R overexpression may be determined in a diagnostic or prognostic assay by evaluating increased levels of the IGF-1R protein present on the surface of a cell (e.g. via an immuno-histochemistry assay; FACS analysis). Alternatively, or additionally, one may measure levels of IGF-1R-encoding nucleic acid or mRNA in the cell, e.g. via fluorescent in situ hybridization; (FISH; see WO98/45479 published October, 1998), Southern blotting, Northern blotting, or polymerase chain reaction (PCR) techniques, such as real time quantitative PCR (RT-PCR). One may also study IGF-1R overexpression by measuring shed antigen in a biological fluid such as serum, e.g, using antibody-based assays (see also, e.g., U.S. Pat. No. 4,933,294 issued Jun. 12, 1990; WO91/05264 published Apr. 18, 1991; U.S. Pat. No. 5,401,638 issued Mar. 28, 1995; and Sias *et al.* J. Immunol. Methods 132: 73-80(1990)). Aside from the above assays, various *in vivo* assays are available to the skilled practitioner. For example, one may expose cells within the body of the patient to an antibody which is optionally labeled with a detectable label, e.g. a radioactive isotope, and binding of the antibody to cells in the patient can be evaluated, e.g. by external scanning for radioactivity or by analyzing a biopsy taken from a patient previously exposed to the antibody. An IGF-1R-expressing cancer includes prostate, bladder, lung, uterine and breast cancer. As used herein, the term "an IGF-1R mediated disorder" is intended to include diseases and other disorders in which the presence of high levels of IGF-IR in a subject suffering from the disorder has been shown to be or is suspected of being either responsible for the pathophysiology of the disorder or a factor that contributes to a worsening of the disorder. Accordingly, a disorder in which high levels of IGF-IR activity is detrimental is a disorder in which inhibition of IGF-IR activity is expected to alleviate the symptoms and/or progression of the disorder. Such disorders may be evidenced, for example, by an increase in the levels of IGF-IR on the cell surface or in increased tyrosine autophosphorylation of IGF-IR in the affected cells or tissues of a

subject suffering from the disorder. The increase in IGF-IR levels may be detected, for example, using an anti-IGF-IR antibody as described above.

"Treating" or "treatment" or "alleviation" refers to both therapeutic treatment and prophylactic or preventative measures; wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented. A subject or mammal is successfully "treated" for a IGF-1R-expressing cancer if, after receiving a therapeutic amount of an anti-IGF-1R antibody according to the methods of the present invention, the patient shows observable and/or measurable reduction in or absence of one or more of the following: reduction of IGF-1R levels, reduction in the number of cancer cells or absence of the cancer cells; reduction in the tumor size; inhibition (i.e., slow to some extent and preferably stop) of cancer cell infiltration into peripheral organs including the spread of cancer into soft tissue and bone; inhibition (i.e., slow to some extent and preferably stop) of tumor metastasis; inhibition, to some extent, of tumor growth; and/or relief to some extent, one or more of the symptoms associated with the specific cancer; reduced morbidity and mortality, and improvement in quality of life issues. To the extent the anti-IGF-1R antibody may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. Reduction of these signs or symptoms may also be felt by the patient.

The above parameters for assessing successful treatment and improvement in the disease are readily measurable by routine procedures familiar to a physician. For cancer therapy, efficacy can be measured, for example, by assessing the time to disease progression (TTP) and/or determining the response rate (RR). For prostate cancer, the progress of therapy can be assessed by routine methods, usually by measuring serum IGF-1R levels. Metastasis can be determined by staging tests and by bone scan and tests for calcium level and other enzymes to determine spread to the bone. CT scans can also be done to look for spread to the pelvis and lymph nodes in the area. Chest X-rays and measurement of liver enzyme levels by known methods are used to look for metastasis to the lungs and liver, respectively. Other routine methods for monitoring the disease include transrectal ultrasonography (TRUS) and transrectal needle biopsy (TRNB).

The term "therapeutically effective amount" refers to an amount of an antibody or a drug effective to "treat" a disease or disorder in a subject or mammal. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. See preceding definition of "treating".

Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents

the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. At.sup.211, I.sup.131, I.sup.125, Y.sup.90, Re.sup.186, Re.sup.188, Sm.sup.153, Bi.sup.212, P.sup.32 and radioactive isotopes of Lu), chemotherapeutic agents e.g. methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents, enzymes and fragments thereof such as nucleolytic enzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, and the various antitumor or anticancer agents disclosed below. Other cytotoxic agents are described below. A tumoricidal agent causes destruction of tumor cells.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an IGF-1R expressing cancer cell, either in vitro or in vivo. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of IGF-1R expressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topoisomerase II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami *et al.* (W B Saunders: Philadelphia, 1995), especially p. 13. The taxanes (paclitaxel and docetaxel) are anticancer drugs both derived from the yew tree. Docetaxel (TAXOTERE®, Rhone-Poulenc Rorer), derived from the European yew, is a semisynthetic analogue of paclitaxel (TAXOL®, Bristol-Myers Squibb). Paclitaxel and docetaxel promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization, which results in the inhibition of mitosis in cells.

"Label" as used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody so as to generate a "labeled" antibody. The label may be detectable by itself (e.g. radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

Antibodies to IGF-1R

The absence in the prior art of information detailing the epitopes on the reference antibody suggests that there was no motivation to generate such data let alone generate anti-IGF-1

receptor antibodies based upon such epitope mapping studies. Consequently, lack of such data impels the conclusion that the prior art not only failed to recognize the advantages of epitope mapping but also failed to envision the advantages of such antibodies. Indeed, in light of the inventors' surprising discoveries disclosed herein, the art is now provided with the knowledge that such specific inhibitory anti-IGF-1 receptor antibodies can be prepared and have distinct advantages.

The invention in its broadest aspect provides details about a specific region on the surface of IGF-1R that is bound by a reference antibody- F50035. The newly discovered discontinuous epitope resides at the C and N terminal portions of the extracellular domain of human IGF-1 receptor.

The epitope specificity of a target antibody can generally be determined by epitope mapping as described, for example, in Ko *et al.*, Brain Research Bulletin, 56:319-329 (2001) and in the Examples below. As a general rule, epitope mapping employs a series of overlapping peptides corresponding to regions on the protein of interest to identify sites which participate in antibody-immunogen determinant interaction, e.g., F50035-IGF-1R. Most commonly, epitope mapping employs peptides of relatively short length to precisely detect linear determinants.

A fast method of epitope mapping known under the trademark "PEIGF-1RN" is based on the simultaneous synthesis of hundreds of overlapping peptides, of lengths of 8 to 14 amino acids, coupled to solid supports. The coupled peptides are tested for their ability to bind antibodies. The PEIGF-1RN approach is effective in localizing linear determinants, but not for the identification of epitopes needed for mimicry of discontinuous effector sites such as the FcεR1 binding site (Meloen *et al.*, Ann Biol Clin, 1991; 49:231-242).

Alternatively, a set of nested and overlapping peptides of multiple lengths ranging from 15 to 60 residues may be employed. These longer peptides can be reliably synthesized by a laborious series of independent solid-phase peptide syntheses, rather than by the rapid and simultaneous PEIGF-1RN syntheses. The resulting set of long nested and overlapping peptides can then be used for analyses of antibody binding in systems such as experimental immunizations and natural infections, to identify long peptides which best present immunodominant determinants, including simple discontinuous epitopes. This method is exemplified by the studies of Wang for the mapping of immunodominant sites from HTLV I/II (U.S. Pat. No. 5,476,765) and HCV (U.S. Pat. No. 5,106,726); and it was used for the selection of a precise position on the gp120 sequence for optimum presentation of an HIV neutralizing epitope (Wang *et al.*, Science, 1991; 254:285-288).

The overall strategy used to map the herein disclosed IGF-1R epitope was performed by the so-called "epitope excision" strategy detailed in JASMS (Jeyarajah S. *et al. JASMS* (1998) 157).

The general strategy used to map the epitopes on human IGF-1 receptor is exemplified here below it being understood that variations and substitutions may be used.

The overall strategy consisted of several steps:

(i) The mAb is immobilized on beads (typically CNBr-activated sepharose beads);

(ii) The antigen in its native form is incubated with the mAb to form the immune complex;

(iii) The complex is subjected to digestion with endoproteinase enzymes in carefully controlled conditions ("limited proteolysis"). While the regions of the antigen which are not in contact with the antibody generate peptide fragments that become free from the immune complex and can be washed away from the beads, the parts of the antigen which correspond to the antigenic region (i.e. the epitope) remain bound to the antibody;

(iv) The bound peptides are identified and characterized by direct analysis of the beads by Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS).

In order to define the smallest region recognized by the antibody (minimal epitope) steps (iii)-(iv) can be repeated on the antigen still bound to the mAb, and after digestion with the first enzyme. Sub-digestions are performed with endoproteinases of different specificity from the one used in the first step of hydrolysis, or with amino- and/or carboxypeptidases, which digest the antigenic peptides starting from their N-or C-terminus, respectively.

The identification of the epitopes on the surface of IGF-1R bound by F50035 will enable one skilled in the art, not only to further characterize this particular antibody but also allow one to identify additional novel antibodies that bind the same epitopic region.

The identification of one or more antibodies that bind(s) to about, substantially, essentially or at the same epitope as the monoclonal antibody F50035 is a straightforward technical matter now that F50035, with its advantageous properties, has been provided and the epitopic region of the IGF-1 receptor bound by the antibody been determined.

Significantly, the subject antibodies identified by any known assay method recognize an epitope residing at the N-terminal region of the alpha chain of IGF-1R defined by Gly_{27}-Arg_{41} and the C-terminal region of the alpha chain between amino acids Tyr_{688}-Arg_{707}. The numbers are relative to the sequence information detailed in Ullrich *et al. infra*. By "recognize" it is meant that the antibodies bind to the IGF-1R protein at the particular epitope detailed herein, preferably the IGF-1R epitope. In many embodiments, the subject antibodies do not bind to any appreciable extent to proteins that do not share a significant degree of homology with the IGF-1R protein.

The terms "that bind to about, substantially or essentially the same, or the same, epitope as" the monoclonal antibody F50035 mean that an antibody "cross-reacts" with the monoclonal antibody F50035. "Cross-reactive antibodies" are those that recognize, bind to or have immunospecificity for substantially or essentially the same, or the same, epitope or "epitopic site" as the monoclonal antibody F50035 such that they are able to effectively compete with the monoclonal antibody F50035 for binding to IGF-1R "F50035-cross-reactive antibodies" are succinctly termed "F50035-like antibodies" and "F50035-based antibodies", and such terms are used interchangeably herein and apply to compositions, uses and methods.

The identification of cross-reactive antibodies can be readily determined using any one of variety of immunological screening assays in which antibody competition can be assessed. All such assays are routine in the art. U.S. Pat. No. 5,660,827, issued Aug. 26, 1997, is specifically incorporated herein by reference for purposes including even further supplementing the present teaching concerning how to make antibodies that bind to the same or substantially the same epitope as a given antibody, such as F50035.

Immunoassays in the competitive binding format can also be used for cross-reactivity determinations. For example, IGF-1R or an immunologically equivalent fragment thereof can be immobilized to a solid support. Proteins are added to the assay that compete for binding of the antisera to the immobilized antigen. The ability of the added proteins to compete for binding of the antisera to the immobilized protein is compared to the ability of IGF-1R to compete with itself. The percent crossreactivity for the above proteins is calculated, using standard calculations. Those antisera with less than 10% cross-reactivity with each of the added proteins listed above are selected and pooled. The cross-reacting antibodies are optionally removed from the pooled antisera by immunoabsorption with the added considered proteins, e.g., distantly related homologues.

The immunoabsorbed and pooled antisera are then used in a competitive binding immunoassay as described above to compare a second protein, thought to be perhaps the protein of this invention, to the immunogen protein (i.e., wild-type IGF-1R (Ullrich *et al.*) or a fragment thereof. In order to make this comparison, the two proteins are each assayed at a wide range of concentrations and the amount of each protein required to inhibit 50% of the binding of the antisera to the immobilized protein is determined. If the amount of the second protein required to inhibit 50% of binding is less than 10 times the amount of the protein encoded by the cDNA encoding the wild type IGF-1R that is required to inhibit 50% of binding, then the second protein is said to specifically bind to the polyclonal antibodies generated to an IGF-1R immunogen.

For example, where the test antibodies to be examined are obtained from different source animals, or are even of a different isotype, a simple competition assay may be employed in which the control (F50035) and test antibodies are admixed (or pre-adsorbed) and applied to a IGF-1 receptor antigen composition. By "IGF-1 receptor antigen composition" is meant any composition that contains a F50035-binding IGF-1 receptor antigen as described herein, such as free IGF-1 receptor. Thus, protocols based upon ELISAs and Western blotting are suitable for use in such simple competition studies.

In certain embodiments, one would pre-mix the control antibodies (F50035) with varying amounts of the test antibodies (e.g., 1:10 or 1:100) for a period of time prior to applying to an antigen composition. In other embodiments, the control and varying amounts of test antibodies can simply be admixed during exposure to the antigen composition. In any event, by using species or isotype secondary antibodies one will be able to detect only the bound control antibodies, the binding of which will be reduced by the presence of a test antibody that recognizes substantially the same epitope

In conducting an antibody competition study between a reference antibody and any test antibody (irrespective of species or isotype), one may first label the control (F50035) with a detectable label, such as, e.g., biotin or an enzymatic (or even radioactive) label to enable subsequent identification. In these cases, one would pre-mix or incubate the labeled control antibodies with the test antibodies to be examined at various ratios (e.g., 1:10 or 1:100) and (optionally after a suitable period of time) then assay the reactivity of the labeled control antibodies and compare this with a control value in which no potentially competing test antibody was included in the incubation.

The assay may again be any one of a range of immunological assays based upon antibody hybridization, and the control antibodies would be detected by means of detecting their label, e.g., using streptavidin in the case of biotinylated antibodies or by using a chromogenic substrate in connection with an enzymatic label (such as 3,3'5,5'-tetramethylbenzidine (TMB) substrate with peroxidase enzyme) or by simply detecting a radioactive label. An antibody that binds to the same epitope as the control antibodies will be able to effectively compete for binding and thus will significantly reduce reference antibody binding, as evidenced by a reduction in bound label.

The reactivity of the (labeled) control antibodies in the absence of a completely irrelevant antibody would be the control high value. The control low value would be obtained by incubating the labeled (F50035) antibodies with unlabelled antibodies of exactly the same type (F50035), when competition would occur and reduce binding of the labeled antibodies. In a test assay, a significant reduction in labeled antibody reactivity in the presence of a test antibody is indicative of a test antibody that recognizes the same epitope, i.e., one that "cross-reacts" with the labeled (F50035) antibody.

A significant reduction is a "reproducible", i.e., consistently observed reduction in binding. A "significant reduction" in terms of the present application is defined as a reproducible reduction (in F50035 binding to IGF-1 receptor in an ELISA) of at least about 70%, about 75% or about 80% at any ratio between about 1:10 and about 1:100. Antibodies with even more stringent cross-blocking activities will exhibit a reproducible reduction (in F50035 binding to IGF-1 receptor in an ELISA or other suitable assay) of at least about 82%, about 85%, about 88%, about 90%, about 92% or about 95% or so at any ratio between about 1:10 and about 1:100. Complete or near-complete cross-blocking, such as exhibiting a reproducible reduction in F50035 binding to IGF-1 receptor of about 99%, about 98%, about 97% or about 96% or so, although by no means required to practice the invention, is certainly not excluded.

To determine if a test/ candidate competing antibody can compete for binding to the same epitope as the epitope bound by the control anti-IGF-1R antibody of the present invention, a cross-blocking assay e.g., a competitive ELISA assay can be performed. In an exemplary competitive ELISA assay, IGF-1R coated on the wells of a microtiter plate is pre-incubated with or without candidate competing antibody and then the biotin-labeled anti-IGF-1R antibody (F50035) is added. The amount of labeled reference antibody bound to the IGF-1R antigen in the wells is measured using avidin-peroxidase conjugate and appropriate substrate. The antibody can be labeled with a radioactive or fluorescent label

or some other detectable and measurable label. The amount of labeled reference antibody that bound to the antigen will have an indirect correlation to the ability of the candidate competing antibody (test antibody) to compete for binding to the same epitope, i.e., the greater the affinity of the test antibody for the same epitope, the less labeled antibody will be bound to the antigen-coated wells. A candidate competing antibody is considered an antibody that binds substantially to the same epitope or that competes for binding to the sane epitope as an anti-IGF-1R antibody of the invention if the candidate antibody can block binding of the IGF-1R antibody by at least 20%, preferably by at least 20-50%, even more preferably, by at least 50% as compared to the control performed in parallel in the absence of the candidate competing antibody (but may be in the presence of a known non-competing antibody). It will be understood that variations of this assay can be performed to arrive at the same quantitative value.

Production of Anti-IGF-1R Antibodies

Antibodies are preferably prepared by standard methods well-known in the art. The subject antibody compositions may be polyclonal, such that a heterogeneous population of antibodies differing by specificity is present, or monoclonal, in which a homogeneous population of identical antibodies that have the same specificity for the IGF-1R epitope are present. Consequently, both monoclonal and polyclonal antibodies are contemplated by the subject invention. Preferably, the subject antibodies are monoclonal antibodies. Other antibody types are also envisioned as described.

The following describes exemplary techniques for the production of the antibodies useful in the present invention. The IGF-1R antigen to be used for production of antibodies may be, e.g., the full length polypeptide or a portion thereof, including a soluble form of IGF-1R or synthetic peptides to selected portions of the protein. Alternatively, cells expressing IGF-1R at their cell surface (e.g. CHO or NIH-3T3 cells transformed to overexpress IGF-1R; prostate or other IGF-1R-expressing tumor cell line), or membranes prepared from such cells can be used to generate antibodies. The nucleotide and amino acid sequences of human and murine IGF-1R are available in the prior art. IGF-1R can be produced recombinantly in and isolated from, bacterial or eukaryotic cells using standard recombinant DNA methodology. IGF-1R can be expressed as a tagged (e.g., epitope tag) or other fusion protein to facilitate isolation as well as identification in various assays. Antibodies or binding proteins that bind to various tags and fusion sequences are available as elaborated below. Other forms of IGF-1R useful for generating antibodies will be apparent to those skilled in the art.

Although methods of making monoclonal and polyclonal antibodies are well known in the art, preferred methods are briefly described herein. Variations of the following methods will be apparent to one of skill in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. For preparation of polyclonal

antibodies, the first step is immunization of the host animal with the immunogen. To increase the immune response of the host animal, the immunogen may be combined with an adjuvant. Suitable adjuvants include alum, dextran, sulfate, large polymeric anions, oil & water emulsions, e.g. Freund's adjuvant, Freund's complete adjuvant, and the like. The immunogen may also be conjugated to synthetic carrier proteins or synthetic antigens. A variety of hosts may be immunized to produce the polyclonal antibodies. Such hosts include without limitation, rabbits, guinea pigs, other rodents such as mice or rats, sheep, goats, primates and the like. The immunogen is administered to the host, usually intradermally, with an initial dosage followed by one or more, usually at least two, additional booster dosages. Following immunization, the blood from the host is collected, followed by separation of the serum from the blood cells. The Ig present in the resultant antiserum may be further fractionated using known methods, such as ammonium salt fractionation, DEAE chromatography, and the like.

Briefly, animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 .mu.g or 5 .mu.g of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later, the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later, the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

As with the preparation of polyclonal antibodies, the first step in preparing monoclonal antibodies specific for an epitope within the IGF-1R, is to immunize a suitable host. Suitable hosts include rats, hamsters, mice, monkeys and the like, and are preferably mice. Monoclonal antibodies may be generated using the hybridoma method described by Kohler *et al.*, Nature, 256:495 (1975) or by recombinant DNA methods, such as those described in U.S. Pat. No. 4,816,567.

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as described above to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. After immunization, lymphocytes are isolated and then fused with a myeloma cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium which medium preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells (also referred to as fusion partner). For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the selective culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and

thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred fusion partner myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a selective medium that selects against the unfused parental cells. Preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 and derivatives e.g., X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromycloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 (1984); and Brodeur *et al.*, Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis described in Munson *et al.*, Anal Biochem., 107:220 (1980).

Once hybridoma cells that produce antibodies of the desired specificity, affinity, and/or activity are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal e.g, by i.p. injection of the cells into mice.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such as, for example, affinity chromatography (e.g., using protein A or protein G-Sepharose) or ion-exchange chromatography, hydroxylapatite chromatography, gel electrophoresis, dialysis, etc.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as E. coli cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al.*, Curr. Opinion in Immunol., 5:256-262 (1993) and Pluckthun, Immunol. Revs., 130:151-188 (1992).

In a further embodiment, monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, Nature,

348:552-554 (1990). Clackson *et al.*, Nature, 352:624-628 (1991) and Marks *et al.*, J. Mol. Biol., 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.*, Bio/Technology, 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, Nuc. Acids. Res., 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA that encodes the antibody may be modified to produce chimeric or fusion antibody polypeptides, for example, by substituting human heavy chain and light chain constant domain (C_H and C_L) sequences for the homologous murine sequences (U.S. Pat. No. 4,816,567; and Morrison, *et al.*, Proc. Natl. Acad. Sci. USA, 81:6851 (1984)), or by fusing the immunoglobulin coding sequence with all or part of the coding sequence for a non-immunoglobulin polypeptide (heterologous polypeptide). The non-immunoglobulin polypeptide sequences can substitute for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized Antibodies

An alternative embodiment of the invention proposes preparing humanized antibodies that recognize the same epitope on IGF-1R as the reference antibody F50035. Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, Nature, 321:522-525 (1986); Reichmann *et al.*, Nature, 332:323-327 (1988); Verhoeyen *et al.*, Science, 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity and HAMA response (human anti-mouse antibody) when the antibody is intended for human therapeutic use. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human V domain sequence which is closest to that of the rodent is identified and the human framework region (FR) within it accepted for the humanized antibody

(Sims *et al.*, J. Immunol., 151:2296 (1993); Chothia *et al.*, J. Mol. Biol., 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.*, Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta *et al.*, J. Immunol., 151:2623 (1993)).

Importantly, the antibodies should be humanized with retention of high binding affinity for the antigen and other favorable biological properties. Towards this end, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Given the epitopic region detailed herein, three-dimensional conformational structures can be constructed via readily available means. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

Various forms of a humanized anti-IGF-1R antibody are contemplated. For example, the humanized antibody may be an antibody fragment, such as a Fab, which is optionally conjugated with one or more cytotoxic agent(s) in order to generate an immunoconjugate. Alternatively, the humanized antibody may be an intact antibody, such as an intact IgG1 antibody.

(iv) Human Antibodies

In an alternative embodiment, fully human antibodies, those that compete with the reference antibody for binding the IGF-1R epitope may also be made.

It is now possible to produce transgenic animals (e.g. mice) that are capable, upon immunization, of producing a repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array into such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits *et al.*, Proc. Natl. Acad. Sci. USA, 90:2551 (1993); Jakobovits *et al.*, Nature, 362:255-258 (1993); Bruggemann *et al.*, Year in Immuno., 7:33 (1993); U.S. Pat. Nos. 5,545,806, 5,569,825, 5,591,669 (all of GenPharm); U.S. Pat. No. 5,545,807; and WO 97/17852.

Mendez *et al.* (Nature Genetics 15: 146-156 [1997]) have further improved the

technology and have generated a line of transgenic mice designated as "Xenomouse II" that, when challenged with an antigen, generates high affinity fully human antibodies. This was achieved by germ-line integration of megabase human heavy chain and light chain loci into mice with deletion into endogenous J_H segment as described above. The Xenomouse II harbors 1,020 kb of human heavy chain locus containing approximately 66 V_H genes, complete D_H and J_H regions and three different constant regions (.mu., .delta. and .chi.), and also harbors 800 kb of human .kappa. locus containing 32 V.kappa. genes, J.kappa. segments and C.kappa. genes. The antibodies produced in these mice closely resemble that seen in humans in all respects, including gene rearrangement, assembly, and repertoire. The human antibodies are preferentially expressed over endogenous antibodies due to deletion in endogenous J_H segment that prevents gene rearrangement in the murine locus.

An alternative approach proposes using phage display technology (McCafferty *et al.*, Nature 348, 552-553 [1990]) to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats, reviewed in, e.g., Johnson, Kevin S. and Chiswell, David J., Current Opinion in Structural Biology 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson *et al.*, Nature, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks *et al.*, J. Mol. Biol. 222:581-597 (1991), or Griffith *et al.*, EMBO J. 12:725-734 (1993). See, also, U.S. Pat. Nos. 5,565,332 and 5,573,905.

Human antibodies may also be generated by *in vitro* activated B cells (see U.S. Pat. Nos. 5,567,610 and 5,229,275).

(v) Antibody Fragments

Sometimes, it is more advantageous to using antibody fragments specific for the IGF-1R epitope detailed herein, rather than whole antibodies. The smaller size of the fragments allows for rapid clearance, and may lead to improved access to solid tumors.

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto *et al.*, Journal of Biochemical and Biophysical Methods 24:107-117 (1992); and Brennan *et al.*, Science, 229:81 (1985)). However, these fragments can now be produced directly by recombinant

host cells. "Antibody fragments" when used herein refer to a portion of an intact antibody, such as the antigen binding or variable region and may include single-chain antibodies, Fab, Fab', F(ab')2 and Fv fragments, diabodies, linear antibodies, and multispecific antibodies generated from portions of intact antibodies.

Fab, Fv and ScFv antibody fragments can all be expressed in and secreted from E. coli, thus allowing the facile production of large amounts of these fragments. Antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from E. coli and chemically coupled to form $F(ab')_2$ fragments (Carter *et al.*, Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Fab and $F(ab')_2$ fragment with increased in vivo half-life comprising a salvage receptor binding epitope residues are described in U.S. Pat. No. 5,869,046. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Pat. Nos. 5,571,894; and 5,587,458. Fv and sFv are the only species with intact combining sites that are devoid of constant regions; thus, they are suitable for reduced nonspecific binding during in vivo use. sFv fusion proteins may be constructed to yield fusion of an effector protein at either the amino or the carboxy terminus of an sFv. See Antibody Engineering, ed. Borrebaeck, supra. The antibody fragment may also be a "linear antibody", e.g., as described in U.S. Pat. No. 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

(vi) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the IGF-1R protein. Other such antibodies may combine an IGF-1R binding site with a binding site for another protein. Alternatively, an anti-IGF-1R arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), so as to focus and localize cellular defense mechanisms to the IGF-1R-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express IGF-1R. These antibodies possess a IGF-1R-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

An exemplary method for preparing a bispecific anti-ErbB2/anti-FcγRIII antibody is described in WO 96/16673. U.S. Pat. No. 5,837,234 discloses a bispecific anti-ErbB2/anti-FcγRI antibody. A bispecific anti-ErbB2/Fc α antibody is shown in WO98/02463. U.S. Pat. No. 5,821,337 teaches a bispecific anti-ErbB2/anti-CD3 antibody.

Methods for making bispecific antibodies are known in the art. Traditional production of

full length bispecific antibodies is based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al.*, Nature, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker *et al.*, EMBO J., 10:3655-3659 (1991).

An alternative approach proposes fusing antibody variable domains with the desired binding specificities (antibody-antigen combining sites) to immunoglobulin constant domain sequences. Preferably, the fusion is with an Ig heavy chain constant domain, comprising at least part of the hinge, C_H 2, and C_H 3 regions. It is preferred to have the first heavy-chain constant region (C_H1) containing the site necessary for light chain bonding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host cell. This provides for greater flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yield of the desired bispecific antibody. It is, however, possible to insert the coding sequences for two or all three polypeptide chains into a single expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios have no significant affect on the yield of the desired chain combination.

Preferably, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et at., Methods in Enzymology, 121:210 (1986).

According to another approach described in U.S. Pat. No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H 3 domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

It is noted that bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980 along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments are available in the prior art. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al.*, Science, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent, sodium arsenite, to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Significant progress has facilitated the direct recovery of Fab'-SH fragments from E. coli, which can be chemically coupled to form bispecific antibodies. Shalaby *et al.*, J. Exp. Med., 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from E. coli and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. See Kostelny *et al.*, J. Immunol., 148(5):1547-1553 (1992).

The "diabody" technology described by Hollinger *et al.*, Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993) provides an alternative mechanism for making bispecific antibody fragments. The fragments comprise a V_H connected to a V_L by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, J. Immunol., 152:5368 (1994).

(vii) Multivalent Antibodies

A multivalent antibody may be internalized (and/or catabolized) faster than a bivalent antibody by a cell expressing an antigen to which the antibodies bind. Preferably, the antigen is the IGF-

1R carrying the epitope derailed herein. As such, one embodiment of the invention contemplates multivalent antibodies (which are other than of the IgM class) with three or more antigen binding sites (e.g. tetravalent antibodies), which can be readily produced by recombinant expression of nucleic acid encoding the polypeptide chains of the antibody. The multivalent antibody can comprise a dimerization domain and three or more antigen binding sites. The preferred dimerization domain comprises (or consists of) an Fc region or a hinge region.

(viii) Other Amino Acid Sequence Modifications

Alternative embodiments of the invention propose anti-IGF-1R antibodies that recognize the IGF-1R epitope having other amino acid sequence modification(s) contained therein. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the anti-IGF-1R antibody are prepared by introducing appropriate nucleotide changes into the anti-IGF-1R antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the anti-IGF-1R antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the anti-IGF-1R antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the anti-IGF-1R antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells in Science, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with IGF-1R antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed anti-IGF-1R antibody variants are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an anti-IGF-1R antibody. with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the anti-IGF-1R antibody molecule include the fusion to the N- or C-terminus of the anti-IGF-1R antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

Another type of variant is an amino acid substitution variant. These variants have at least

one amino acid residue in the anti-IGF-1R antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 1

	Amino Acid Substitutions	
Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	Lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; arg	Gln
Asp (D)	glu; asn	Glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	Asp
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	Arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala ; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	Tyr
Pro (P)	ala	ala
Ser (S)	thr	Thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	Tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala ; norleucine	leu

Substantial modifications in the biological properties of the antibody can be accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the anti-IGF-1R antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human IGF-1R. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Nucleic acid molecules encoding amino acid sequence variants of the subject anti-IGF-1R antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the anti-IGF-1R antibody.

At times, it may be desirable to modify the antibody of the invention with respect to effector function, e.g. so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al.*, J. Exp Med. 176:1191-1195 (1992) and Shopes, B. J. Immunol. 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al.* Cancer Research 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al.* Anti-Cancer Drug Design 3:219-230 (1989).

Likewise, to increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in U.S. Pat. No. 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

(ix) Screening for Antibodies with the Desired Properties

Once antibodies that bind the same epitope as the reference antibody have been generated, one may further select antibodies with certain biological characteristics, as desired.

For example, the growth inhibitory effects of an anti-IGF-1R antibody of the invention may be assessed by methods known in the art, e.g., using cells which express IGF-1R either endogenously or following transfection with the IGF-1R gene. For example, the tumor cell lines and IGF-1R-transfected cells may be treated with an anti-IGF-1R monoclonal antibody of the invention at various concentrations for a few days (e.g., 2-7) days and stained with crystal violet or MTT or analyzed by some other colorimetric assay. Another method of measuring proliferation would be by comparing ^{3}H-thymidine uptake by the cells treated in the presence or absence an anti-IGF-1R antibody of the invention. After antibody treatment, the cells are harvested and the amount of radioactivity incorporated into the DNA quantitated in a scintillation counter. Appropriate positive controls include treatment of a selected cell line with a growth inhibitory antibody known to inhibit growth of that cell line. Growth inhibition of tumor cells *in vivo* can be determined in various ways known ton to one skilled in the art. For example, a tumor cell that over-expresses IGF-1R may be used. Preferably, the anti-IGF-1R antibody will inhibit cell proliferation of a IGF-1R-expressing tumor cell *in vitro* or *in vivo* by about 25-100% compared to the untreated tumor cell, more preferably, by about 30-100%, and even more preferably by about 50-100% or 70-100%, at an antibody concentration of about 0.5 to 30 .mu.g/ml. Growth inhibition can be measured at an antibody concentration of about 0.5 to 30 .mu.g/ml or about 0.5 nM to 200 nM in cell culture, where the growth inhibition is determined 1-10 days after exposure of the tumor cells to the antibody. The antibody is growth inhibitory in vivo if administration of the anti-IGF-1R antibody at about 1 .mu.g/kg to about 100 mg/kg body weight results in reduction in tumor size or tumor cell proliferation within about 5 days to 3 months from the first administration of the antibody, preferably within about 5 to 30 days.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., propidium iodide (PI), trypan blue or 7AAD uptake may be assessed relative to control. A PI uptake assay can be performed in the absence of complement and immune effector cells. IGF-1R-expressing tumor cells are incubated with medium alone or medium containing the appropriate monoclonal antibody at e.g, about 10 .mu./ml. The cells are incubated for a 3 day time period. Following each treatment, cells are washed and aliquoted into 35 mm strainer-capped 12.times.75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 .mu.g/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake may be selected as cell death-inducing antibodies.

To screen for antibodies which bind to an epitope on IGF-1R bound by an antibody of interest, a routine cross-blocking assay such as that described in Antibodies, A Laboratory Manual, Cold

Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. This assay can be used to determine if a test antibody binds the same site or epitope as an anti-IGF-1R antibody of the invention. Alternatively, or additionally, epitope mapping can be performed by methods known in the art. For example, the antibody sequence can be mutagenized such as by alanine scanning, to identify contact residues. The mutant antibody is initially tested for binding with polyclonal antibody to ensure proper folding. In a different method, peptides corresponding to different regions of IGF-1R can be used in competition assays with the test antibodies or with a test antibody and an antibody with a characterized or known epitope.

(x) Immunoconjugates

The invention also pertains to therapy with immunoconjugates comprising an antibody conjugated to an anti-cancer agent such as a cytotoxic agent or a growth inhibitory agent. Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above.

Conjugates of an antibody, i.e., invention antibody and one or more small molecule toxins, such as a calicheamicin, maytansinoids, a trichothene, and CC1065, and the derivatives of these toxins that have toxin activity, are also contemplated herein. Other antitumor agents that can be conjugated to the anti-IGF-1R antibodies of the invention include BCNU, streptozoicin, vincristine and 5-fluorouracil, the family of agents known collectively LL-E33288 complex described in U.S. Pat. Nos. 5,053,394; 5,770,710 as well as esperamicins (U.S. Pat. No. 5,877,296).

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published Oct. 28, 1993.

The present invention further contemplates an immunoconjugate formed between an antibody and a compound with nucleolytic activity (e.g. a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

For selective destruction of the tumor, the antibody may comprise a highly radioactive atom. A variety of radioactive isotopes are available for the production of radioconjugated anti-IGF-1R antibodies. Examples include At.sup.211, I.sup.131, I.sup.125, Y.sup.90, Re.sup.186, Rc.sup.188, Sm.sup.153, Bi.sup.212, P.sup.32, Pb.sup.212 and radioactive isotopes of Lu. When the conjugate is used for diagnosis, it may comprise a radioactive atom for scintigraphic studies, for example tc.sup.99m or I.sup.123, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, MRI), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

The radio- or other labels may be incorporated in the conjugate in known ways. For

example, the peptide may be biosynthesized or may be synthesized by chemical amino acid synthesis using suitable amino acid precursors involving, for example, fluorine-19 in place of hydrogen. Labels such as tc.sup.99m or I.sup.123, .Re.sup.186, Re.sup.188 and In.sup.111 can be attached via a cysteine residue in the peptide. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker *et al* (1978) Biochem. Biophys. Res. Commun. 80: 49-57 can be used to incorporate iodine-123. "Monoclonal Antibodies in Immunoscintigraphy" (Chatal,CRC Press 1989) describes other methods in detail.

Conjugates of the subject antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), succinimidyl-4(N-maleimidomethyl)cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl)hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.* Science 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, Cancer Research 52: 127-131 (1992); U.S. Pat. No. 5,208,020) may be used.

Alternatively, a fusion protein comprising the anti-IGF-1R antibody and cytotoxic agent may be made, e.g. by recombinant techniques or peptide synthesis. The length of DNA may comprise respective regions encoding the two portions of the conjugate either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the conjugate.

In an alternative embodiment, the subject antibody may be conjugated to a "receptor" (such streptavidin) for ultimate use in tumor pre-targeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(xi) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278. The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form. Such enzymes are readily apparent to one skilled in the art. These include, but are not limited to, alkaline

phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs etc. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, Nature 328: 457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The subject enzymes can be covalently bound to the anti-IGF-1R antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art(see, e.g., Neuberger *et al.*, Nature, 312: 604-608 (1984).

(xii) Other Antibody Modifications

Other modifications to the invention antibodies are possible and contemplated by the invention. For example, an invention antibody may be linked to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol. The antibody also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate)microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Oslo, A., Ed., (1980).

The invention anti-IGF-1R antibodies according to any one or more of the above embodiments may also be formulated as immunoliposomes. A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al.*, Proc. Natl. Acad. Sci. USA, 82:3688 (1985); Hwang *et al.*, Proc. Natl Acad. Sci. USA, 77:4030 (1980); U.S. Pat. Nos. 4,485,045 and 4,544,545; and WO97/38731 published Oct. 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Nucleic Acid Molecules

The invention also provides isolated nucleic acid encoding the anti-IGF-1R antibodies of the invention, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibodies. Nucleic acid molecules encoding amino acid sequence variants of anti-IGF-IR antibody are also provided. The nucleic acid molecules can be produced by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of humanized anti- IGF-IR antibody.

The antibodies according to the invention are preferably produced by recombinant means. Such methods are widely known in the state of the art and comprise protein expression in prokaryotic and eukaryotic cells with subsequent isolation of the antibody polypeptide and usually purification to a pharmaceutically acceptable purity. See Makrides, S. C., Protein Expr. Purif. 17 (1999) 183-202; Geisse, S., *et al.*, Protein Expr. Purif. 8 (1996) 271-282; Kaufman, R. J., Mol. Biotechnol. 16 (2000) 151- 161; Werner, R. G., Drug Res. 48 (1998) 870- 880. For the protein expression, nucleic acids encoding light and heavy chains or fragments thereof are inserted into expression vectors by standard methods. Expression is performed in appropriate prokaryotic or eukaryotic host cells like CHO cells, NS0 cells, SP2/0 cells, HEK293 cells, COS cells, yeast, or E. coli cells, and the antibody is recovered from the cells (supernatant or cells after lysis).

Host cells, preferably eukaryotic cells such as CHO cell or COS cells, are transformed with the above-described expression or cloning vectors for anti-IGF-1R antibody production and cultured according to well-established procedures.

Expression in NS0 cells is described by, e.g., Barnes, L. M., *et al.*, Cytotechnology 32 (2000) 109-123; and Barnes, L. M., *et al.*, Biotech. Bioeng. 73 (2001) 261-270. Transient expression is described by, e.g., Durocher, Y., *et al.*, Nucl. Acids. Res. 30 (2002) E9. Cloning of variable domains is described by Orlandi, R., *et al.*, Proc. Natl. Acad. Sci. USA 86 (1989) 3833-3837; Carter, P., *et al.*, Proc. Natl. Acad. Sci. USA 89 (1992) 4285-4289; and Norderhaug, L., *et al.*, J. Immunol. Methods 204 (1997) 77-87. A preferred transient expression system (HEK 293) is described by Schlaeger, E.-J., and Christensen, K., in Cytotechnology 30 (1999) 71-83 and by Schlaeger, E.-J., in J. Immunol. Methods 194 (1996) 191-199.

Diagnostic Applications

The subject antibodies, or antigen binding fragments thereof will find use in immunoassays that are capable of providing for the detection of an a protein carrying an IGF-1R epitope in a sample. In such assays, the sample suspected of comprising an IGF-1R molecule carrying the IGF-1R epitope of interest will typically be obtained from a subject, such as a human subject, suspected of suffering from the disease of interest or at risk for developing the disease of interest. The sample is

generally a physiological sample from the patient such as blood or tissue. Depending on the nature of the sample, it may or may not be pretreated prior to assay, as will be apparent to one of skill in the art.

A number of different immunoassay formats are known in the art and may be employed in detecting the presence of protein of interest in a sample. Immunoassays of interest include Western blots on protein gels or protein spots on filters, where the antibody is labeled, as is known in the art. A variety of protein labeling schemes are known in the art and may be employed, the particular scheme and label chosen being the one most convenient for the intended use of the antibody, e.g. immunoassay. Examples of labels include labels that permit both the direct and indirect measurement of the presence of the antibody. Examples of labels that permit direct measurement of the antibody include radiolabels, such as ^{3}H or ^{125}I, fluorescent dyes, beads, chemiluminescers and colloidal particles. Examples of labels which permit indirect measurement of the presence of the antibody include enzymes where a substrate may be provided for a colored or fluorescent product. For example, the antibodies may be labeled with a covalently bound enzyme capable of providing a detectable product signal after addition of suitable substrate. Instead of covalently binding the enzyme to the antibody, the antibody may be modified to comprise a first member of specific binding pair which specifically binds with a second member of the specific binding pair that in conjugated to the enzyme, e.g. the antibody may be covalently bound to biotin and the enzyme conjugate to streptavidin. Examples of suitable enzymes for use in conjugates include horseradish peroxidase, alkaline phosphatase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art.

Other immunoassays include those based on a competitive format, as are known in the art. One such format would be where a solid support is coated with the pIGF-1R protein or a fragment thereof that carries the IGF-1R epitope. Labeled antibody is then combined with a sample suspected of comprising protein of interest to produce a reaction mixture which, following sufficient incubation time for binding complexes to form, is contacted with the solid phase bound protein. The amount of labeled antibody which binds to the solid phase will be proportional to the amount of protein in the sample, and the presence of protein may therefore be detected. Other competitive formats that may be employed include those where the sample suspected of comprising protein is combined with a known amount of labeled protein and then contacted with a solid support coated with antibody specific for the protein. Such assay formats are known in the art and further described in both Guide to Protein Purification, *supra*, and Antibodies, A Laboratory Manual (Cold Springs Harbor Press (Cold Springs Harbor, N.Y. 1989)).

Those immunoassays involving solid supports, the solid support may be any compositions to which antibodies or fragments thereof can be bound, which is readily separated from soluble material, and which is otherwise compatible with the overall immunoassay method. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports to which the receptor is bound include beads, e.g. magnetic beads, membranes and microtiter

plates. These are typically made of glass, plastic (e.g. polystyrene), polysaccharides, nylon or nitrocellulose. Microtiter plates are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples.

Before adding patient samples or fractions thereof, the non-specific binding sites on the insoluble support i.e. those not occupied by the first antibody, are generally blocked. Preferred blocking agents include non-interfering proteins such as bovine serum albumin, casein, gelatin, and the like. Alternatively, detergents, such as Tween, NP40 or TX100 may be used at non-interfering concentrations.

It is particularly convenient in a clinical setting to perform the immunoassay in a self-contained apparatus, and such devices are provided by the subject invention. A number of such devices and methods for their use are known in the art. The apparatus will generally employ a continuous flow-path over a suitable filter or membrane, and will have at least three regions, a fluid transport region, a sample region, and a measuring region. The sample region is prevented from fluid transfer contact with the other portions of the flow path prior to receiving the sample. After the sample region receives the sample, it is brought into fluid transfer relationship with the other regions, and the fluid transfer region contacted with fluid to permit a reagent solution to pass through the sample region and into the measuring region. The measuring region may have bound to it a first antibody. The second, labeled antibody combined with the assayed sample is introduced and the sandwich assay performed as above.

Thus, an embodiment of the invention provides anti-IGF-IR antibody that may be used to detect IGF-IR in a biological sample *in vitro* or *in vivo*. The anti-IGF-IR antibody may be used in a conventional immunoassay, including, without limitation, an ELISA, an RIA, FACS, tissue immunohistochemistry, Western blot or immunoprecipitation. The anti-IGF-IR antibody of the invention may be used to detect IGF-IR from humans. In another embodiment, the anti-IGF-IR antibody (or antibodies) of the invention may be used to detect IGF-IR from Old World primates such as cynomolgus and rhesus monkeys, chimpanzees and apes, particularly those that carry the IGF-1R epitope detailed herein. The method for detecting anti-IGF-IR in a biological sample comprises contacting a biological sample with an anti-IGF-IR antibody of the invention and detecting the bound antibody bound to anti-IGF-IR, to detect the IGF-IR in the biological sample. In another embodiment, the anti-IGF-IR antibody is directly labeled with a detectable label. In yet another embodiment, the anti-IGF-IR antibody (the first antibody) is unlabeled and a second antibody or other molecule that can bind the anti-IGF-IR antibody is labeled. As is well known to one of skill in the art, a second antibody is chosen that is able to specifically bind the specific species and class of the first antibody. For example, if the anti-IGF-IR antibody is a human IgG, then the secondary antibody may be an anti-human-IgG. Other molecules that can bind to antibodies include, without limitation, Protein A and Protein G, both of which are available commercially, e.g., from Pierce Chemical Co.

Suitable labels for the antibody or secondary are well known and include, without limitation, various enzymes, prosthetic groups, fluorescent materials, luminescent materials, magnetic agents and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline

phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; an example of a magnetic agent includes gadolinium; and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

In an alternative embodiment, IGF-IR can be assayed in a biological sample by a competition immunoassay utilizing IGF-IR standards labeled with a detectable substance and an unlabeled anti-IGF-IR antibody. In this assay, the biological sample, the labeled IGF-IR standards, e.g., F50035 and the anti-IGF-IR antibody (candidate antibody) are combined and the amount of labeled IGF-IR standard bound to the unlabeled antibody is determined. The amount of IGF-IR in the biological sample is inversely proportional to the amount of labeled IGF-IR standard bound to the anti-IGF-IR antibody.

One may use the immunoassays disclosed above for a number of purposes. In one embodiment, the anti-IGF-IR antibody identified according to a method of the invention may be used to detect IGF-IR in cells in cell culture. In a preferred embodiment, the anti-IGF-IR antibody may be used to determine the level of tyrosine phosphorylation, tyrosine autophosphorylation of IGF-IR, and/or the amount of IGF-IR on the cell surface after treatment of the cells with various compounds. This method can be used to test compounds that may be used to activate or inhibit IGF-IR. In this method, one sample of cells is treated with a test compound for a period of time while another sample is left untreated. If tyrosine autophosphorylation is to be measured, the cells are lysed and tyrosine phosphorylation of the IGF-IR is measured using an immunoassay described above or as described previously using an ELISA. If the total level of IGF-IR is to be measured, the cells are lysed and the total IGF-IR level is measured using one of the immunoassays described above.

A preferred immunoassay for determining IGF-IR tyrosine phosphorylation or for measuring total IGF-IR levels is an ELISA or Western blot. If only the cell surface level of IGF-IR is to be measured, the cells are not lysed, and the cell surface levels of IGF-IR are measured using one of the immunoassays described above. A preferred immunoassay for determining cell surface levels of IGF-IR includes the steps of labeling the cell surface proteins, e.g. wild type IGF-1R carrying the IGF-1R epitope or a recombinanat protein carrying the same epitope with a detectable label, such as biotin or ^{125}I, immunoprecipitating the IGF-IR with an anti-IGF-IR antibody and then detecting the labeled IGF-IR.

Another preferred immunoassay for determining the localization of IGF-IR, e.g., cell surface levels, is by using immunohistochemistry. Methods such as ELISA, RIA, Western blot, immunohistochemistry, cell surface labeling of integral membrane proteins and immunoprecipitation are well known in the art. See, e.g., Harlow and Lane, *supra*. In addition, the immunoassays may be scaled up for high throughput screening in order to test a large number of compounds for either activation or inhibition of IGF-IR.

The anti-IGF-IR antibody of the invention may also be used to determine the levels of IGF-IR in a tissue or in cells derived from the tissue. In a preferred embodiment, the tissue is a diseased tissue. In a more preferred embodiment, the tissue is a tumor or a biopsy thereof. In a preferred embodiment of the method, a tissue or a biopsy thereof is excised from a patient. The tissue or biopsy is then used in an immunoassay to determine, e.g., IGF-IR levels, cell surface levels of IGF-IR, levels of tyrosine phosphorylation of IGF-IR, or localization of IGF-IR by the methods discussed above. The method can be used to determine if a tumor expresses IGF-IR at a high level.

The above-described diagnostic method can be used to determine whether a tumor expresses high levels of IGF-IR, which may be indicative that the tumor will respond well to treatment with anti-IGF-IR antibody. The diagnostic method may also be used to determine whether a tumor is potentially cancerous, if it expresses high levels of IGF-IR, or benign, if it expresses low levels of IGF-IR. As well, the diagnostic method may also be used to determine whether treatment with anti-IGF-IR antibody is causing a tumor to express lower levels of IGF-IR and/or to express lower levels of tyrosine autophosphorylation, and thus can be used to determine whether the treatment is successful.

The proposed method to determine whether an anti-IGF-IR antibody decreases tyrosine phosphorylation comprises the steps of measuring the level of tyrosine phosphorylation in a cell or tissue of interest, incubating the cell or tissue with an anti-IGF-IR antibody of the invention or antigen-binding portion thereof, then re-measuring the level of tyrosine phosphorylation in the cell or tissue. The tyrosine phosphorylation of IGF-IR or of another protein(s) may be measured. The diagnostic method may also be used to determine whether a tissue or cell is not expressing high enough levels of IGF-IR or high enough levels of activated IGF-IR, which may be the case for individuals with dwarfism, osteoporosis or diabetes. A diagnosis that levels of IGF-IR or active IGF-IR are too low could be used for treatment with activating anti-IGF-IR antibodies, IGF-I or other therapeutic agents for increasing IGF-IR levels or activity.

Based on the ability of the antibody of the present invention to down regulate IGF-1R on peripheral lymphocytes, a "biomarker strategy" may be employed to monitor the expression of IGF-1 R on circulating tumor and/or normal cells from patients treated with the antibody of the invention. These cells can include but are not limited to CD19+ cells, and may also include all white blood cells such as monocytes, granulocytes, and lymphocytes.

Screening to Identify Compounds with a Desired Biological Activity

After production of the desired antibodies to a target immunogen, they may be screened for desirable biological properties, such as high affinity binding to the desired antigen, e.g., IGF-1R that is also bound by the reference antibody and carries the IGF-1R epitope, the ability to prevent activation of an IGF-1R mediated signaling cascade associated with the reference antibody upon its binding IGF-1R etc.

The subject antibodies, including immunologically equivalent binding fragments thereof

will also find use in screening applications designed to identify agents or compounds that are capable of modulating, e.g. inhibiting, the binding interaction between the protein to which the antibody binds and a cellular target. For example, the subject antibodies will find use in screening assays that identify compounds capable of modulating the interaction between IGF-1R and its cellular targets, e.g., IGF-1 and *IGF-II*.

In such assays, the subject antibody is contacted with IGF-1R or its extracellular domain carrying the IGF-1R epitope in the presence of a candidate modulation agent and any resultant binding complexes between the antibody and IGF-1R are detected. The results of the assay are then compared with a control. Those agents that change the amount of binding complexes that are produced upon contact are identified as agents that modulate the binding activity of IGF-1R and therefore are potential therapeutic agents. Of interest in many embodiments is the identification of agents that inhibit, at least to some extent, the binding of IGF-1R with its target. In many assays, at least one of the protein or antibody is attached to a solid support and at least one of these members is labeled, where supports and labels are described *supra*.

In other assays, the ability of a candidate compound to disrupt or enhance the biological activity of an anti-IGF-1R antibody is measured. For example, the ability of a candidate compound to prevent tyrosine activation or enhance cell death, or mimic other biological properties normally produced by an anti-IGF-1R antibody including the reference antibody – F50035 may be measured.

A variety of different candidate agents may be screened by the above screening methods. Candidate agents encompass numerous chemical classes and also include biological moieties, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate agents are also found among biomolecules including polypeptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Antibodies that inhibit IGF-IR tyrosine phosphorylation and receptor levels *in vivo* are also provided by the present invention. The levels of tyrosine phosphorylation may be measured by any method known in the art. For example, in one embodiment, administration of IGF-IR antibody to an animal causes a reduction in IGF- IR phosphotyrosine signal in IGF-IR-expressing tumors. In a preferred embodiment, the IGF-IR antibody causes a reduction in phosphotyrosine signal by at least 20%. In a more preferred embodiment, the IGF-IR antibody causes a decrease in the phosphotyrosine signal by at least 50%, more preferably 60%. In an even more preferred embodiment, the antibody causes a decrease in phosphotyrosine signal of at I least 70%, more preferably 80%, even more preferably 90%.

In a preferred embodiment, the antibody is administered approximately 24 hours before the levels of tyrosine phosphorylation are measured. Preferably, the antibody binds the same epitope on IGF-1R as the reference antibody, e.g., IGF-1R epitope.

In an alternative embodiment, administration of IGF-IR antibody to an animal causes a reduction in IGF-IR levels in IGF-IR-expressing tumors. In a preferred embodiment, the IGF-IR antibody

causes a reduction in receptor levels by at least 20% compared to an untreated animal. In a more preferred embodiment, the IGF-IR antibody causes a decrease in receptor levels to at least 50%, more preferably 60% of the receptor levels in an untreated animal. In an even more preferred embodiment, the antibody causes a decrease in receptor levels by at least 70%, more preferably 80%. In a preferred embodiment, the antibody is administered approximately 24 hours before the IGF-IR levels are measured. The IGF-IR levels may be measured by any method known in the art.

In another embodiment, an IGF-IR antibody inhibits tumor cell growth *in vivo*. The tumor cell may be derived from any cell type including, without limitation, epidermal, epithelial, endothelial, leukemia, sarcoma, multiple myeloma, or mesodermal cells. Examples of common tumor cell lines for use in xenograft tumor studies include A549 (non-small cell lung carcinoma) cells, DU-145 (prostate) cells, ; MCF-7 (breast) cells, Colo 205 (colon) cells, 3T3/IGF-IR (mouse fibroblast) cells, NCI H441 cells, HEP G2 (hepatoma) cells, MDA MB 231 (breast) cells, HT-29 (colon) cells, MDA-MB-435s (breast) cells, U266 cells, SH-SY5Y cells, Sk-Mel-2 cells, NCI-H929, RPMI8226, and A431 cells. Preferably, the subject antibody inhibits tumor cell growth as compared to the growth of the tumor in an untreated animal. In a more preferred embodiment, the antibody inhibits tumor cell growth by 50%O In an even more preferred embodiment, the antibody inhibits tumor cell growth by 60%, 65%, 70%, or 75%. In one embodiment, the inhibition of tumor cell growth is measured at least 7 days after the animals have started treatment with the antibody. In a more preferred embodiment, the inhibition of tumor cell growth is measured at least 14 days after the animals have started treatment with the antibody.

In another preferred embodiment, another antineoplastic agent is administered to the animal with the IGF-IR antibody. In a preferred embodiment the antineoplastic agent is able to further inhibit tumor cell growth. Preferably, the antineoplastic agent is adriamycin, taxol, tamoxifen, 5-fluorodeoxyuridine (5-FU) or CP- 358,774.

In a preferred embodiment' the co-administration of an antineoplastic agent and the IGF-IR antibody inhibits tumor cell growth by at least 50%, more preferably 60%, 65%, 70% or 75%, more preferably 80%, 85% or 90% after a period of 22-24 days.

Methods of Treatment – Cancer Immunotherapy

Since IGF-1R is expressed or overexpressed in many cancers, including but not limited to prostate tumors, metastases of prostate tumors, it is a target for cancer immunotherapy. These immunotherapeutic methods include the use of antibody therapy as well as *ex vivo* immunotherapy approaches.

In one approach, the invention provides anti-IGF-1R antibodies of the invention that may be used systemically to treat cancer, such as prostate, bladder and pancreatic cancer. Anti-IGF-1R antibodies may also be useful in the treatment of various other benign and malignant tumors.

Antibodies which bind specifically to the extracellular domain of IGF-1R are preferred, especially those that bind the IGF-1R epitope detailed herein. Antibodies which target the tumor cells but not the surrounding non-tumor cells and tissue are also preferred.

In one aspect, the invention provides a method of treating a patient susceptible to or having a cancer which expresses IGF-1R, comprising administering to said patient an effective amount of an antibody which binds specifically to the extracellular domain of IGF-1R, particularly the IGF-1R epitope. In another approach, the invention provides a method of inhibiting the growth of tumor cells expressing IGF-1R, comprising administering to a patient an antibody which binds specifically to the extracellular domain of IGF-1R, particularly the IGF-1R epitope, in an amount effective to inhibit growth of the tumor cells. IGF-1R mAbs may also be used in a method for selectively inhibiting the growth of or killing a cell expressing IGF-1R antigen comprising reacting a IGF-1R antibody immunoconjugate or immunotoxin with the cell in an amount sufficient to inhibit the growth of or kill the cell.

For example, unconjugated IGF-1R antibody (including monoclonal, polyclonal, chimeric, humanized, fully human and fragments thereof (e.g., recombinant proteins)) may be introduced into a patient such that the antibody binds to IGF-1R on cancer cells and mediates growth inhibition of such cells (including the destruction thereof, and the tumor, by mechanisms which may include complement-mediated cytolysis, antibody-dependent cellular cytotoxicity, altering the physiologic function of IGF-1R, and/or the inhibition of ligand binding or signal transduction pathways. In addition to unconjugated IGF-1R antibodies, fragments thereof, and recombinant proteins of the invention, IGF-1R antibodies conjugated to toxic agents such as ricin may also be used therapeutically to deliver the toxic agent directly to IGF-1R-bearing tumor cells and thereby destroy the tumor.

Cancer immunotherapy using IGF-1R antibodies may follow the teachings generated from various approaches which have been successfully employed with respect to other types of cancer, including but not limited to colon cancer (Arlen *et al.*, 1998, Crit Rev Immunol 18: 133-138), multiple myeloma (Ozaki *et al.*, 1997, Blood 90: 3179-3186; Tsunenari *et al.*, 1997, Blood 90: 2437-2444), gastric cancer (Kasprzyk *et al.*, 1992, Cancer Res 52: 2771-2776), B-cell lymphoma (Funakoshi *et al.*, 1996, J Immunther Emphasis Tumor Immunol 19: 93-101), leukemia (Zhong *et al.*, 1996, Leuk Res 20: 581-589), colorectal cancer (Moun *et al.*, 1994, Cancer Res 54: 6160-6166); Velders *et al.*, 1995, Cancer Res 55: 4398-4403), and breast cancer (Shepard *et al.*, 1991, J Clin Immunol 11: 117-127).

A representative example useful in a clinical setting is administering them in unmodified form, using monoclonal antibodies of the invention which display antitumor activity (e.g., ADCC and CDC activity) and/or internalizing ability *in vitro* and/or in animal models (see, e.g. Helistrom *et al.*, Proc. Natl. Acad. Sci. USA 82:1499-1502 (1985). To detect ADCC and CDC activity monoclonal antibodies can be tested for lysing cultured ^{51}Cr-labeled tumor target cells over a 4-hour incubation period. Target cells are labeled with ^{51}Cr and then can be exposed for 4 hours to a combination of effector cells (in the form of human lymphocytes purified by the use of a lymphocyte-separation

medium) and antibody, which is added in concentrations, e.g., varying between 0.1 .mu.g/ml and 10 .mu.g/ml. The release of ^{51}Cr from the target cells is measured as evidence of tumor-cell lysis (cytotoxicity). Controls include the incubation of target cells alone or with either lymphocytes or monoclonal antibody separately. The total amount of ^{51} Cr that can be released is measured and ADCC is calculated as the percent killing of target cells observed with monoclonal antibody plus effector cells as compared to target cells being incubated alone. The procedure for CDC is identical to the one used to detect ADCC except that human serum, as a source of complement, (diluted 1:3 to 1:6) is added in place of the effector cells.

In the practice of the method of the invention, anti-IGF-1R antibodies capable of inhibiting the growth of cancer cells expressing IGF-1R on the cell surface are administered in a therapeutically effective amount to cancer patients whose tumors express or overexpress IGF-1R. The anti-IGF-1R mAb therapy method of the invention demonstrates remarkable tumor growth inhibition of prostate tumors *in vivo*. Accordingly, the invention provides a specific, effective and long-needed treatment for prostate cancer. The method of the invention may also be useful for the treatment of other cancers which express or overexpress IGF-1R, including but not limited to bladder carcinoma and pancreatic carcinomas, since both of these cancers express elevated levels of IGF-1R. The antibody therapy methods of the invention may be combined with a chemotherapeutic, radiation, and/or other therapeutic regimen.

Patients may be evaluated for the presence and level of IGF-1R overexpression in tumors, preferably using immunohistochemical assessments of tumor tissue, quantitative IGF-1R imaging, or other techniques capable of reliably indicating the presence and degree of IGF-1R expression Immunohistochemical analysis of tumor biopsies or surgical specimens may be preferred for this purpose. Methods for immunohistochemical analysis of tumor tissues are well known in the art. An example of an immunohistochemical analytical technique useful for determining the level of IGF-1R overexpression in a sample is described in the example sections below.

Anti-IGF-1R monoclonal antibodies useful in treating cancer include those which are capable of initiating a potent immune response against the tumor and those which are capable of direct cytotoxicity. In this regard, anti-IGF-1R mAbs may elicit tumor cell lysis by either complement-mediated or antibody-dependent cell cytotoxicity (ADCC) mechanisms, both of which require an intact Fc portion of the immunoglobulin molecule for interaction with effector cell Fc receptor sites or complement proteins. In addition, anti-IGF-1R mAbs which exert a direct biological effect on tumor growth are useful in the practice of the invention. Such mAbs may not require the complete immunoglobulin to exert the effect. Potential mechanisms by which such directly cytotoxic mAbs may act include inhibition of cell growth, modulation of cellular differentiation, modulation of tumor angiogenesis factor profiles, and the induction of apoptosis. The mechanism by which a particular anti-IGF-1R mAb exerts an anti-tumor effect may be evaluated using any number of in vitro assays designed to determine ADCC, ADMMC, complement-mediated cell lysis, and so forth, such as those described in Example 19, below.

The anti-tumor activity of a particular anti-IGF-1R mAb, or combination of anti-IGF-1R mAbs, is preferably evaluated *in vivo* using a suitable animal model. Xenogenic cancer models, wherein human cancer explants or passaged xenograft tissues are introduced into immune compromised animals, such as nude or SCID mice, are particularly appropriate and are known. Examples of xenograft models of human prostate cancer (capable of recapitulating the development of primary tumors, micrometastasis, and the formation of osteoblastic metastases characteristic of late stage disease) are described in Klein *et al.*, 1997, Nature Medicine 3: 402-408 and in PCT Patent Application WO98/16628, Sawyers *et al.*, published Apr. 23, 1998. The examples herein provide detailed experimental protocols for evaluating the anti-tumor potential of anti-IGF-1R mAb preparations in vivo. Other in vivo assays are contemplated, such as those which measure regression of established tumors, interference with the development of metastasis, and the like.

It should be noted that the use of murine or other non-human monoclonal antibodies and chimeric mAbs may induce moderate to strong immune responses in some patients. Accordingly, preferred monoclonal antibodies used in the practice of the therapeutic methods of the invention are those which are either fully human or humanized and which bind specifically to the target IGF-1R antigen with high affinity but exhibit low or no antigenicity in the patient.

The method of the invention contemplate the administration of single anti-IGF-1R mAbs as well as combinations, or "cocktails, of different individual mAbs such as those recognizing different epitopes. Such mAb cocktails may have certain advantages inasmuch as they contain mAbs which bind to different epitopes and/or exploit different effector mechanisms or combine directly cytotoxic mAbs with mAbs that rely on immune effector functionality. Such mAbs in combination may exhibit synergistic therapeutic effects. In addition, the administration of anti-IGF-1R mAbs may be combined with other therapeutic agents, including but not limited to various chemotherapeutic agents, androgen-blockers, and immune modulators (e.g., IL-2, GM-CSF). The anti-IGF-1R mAbs may be administered in their "naked" or unconjugated form, or may have therapeutic agents conjugated to them.

The anti-IGF-1R monoclonal antibodies used in the practice of the method of the invention may be formulated into pharmaceutical compositions comprising a carrier suitable for the desired delivery method. Suitable carriers include any material which when combined with the anti-IGF-1R mAbs retains the anti-tumor function of the antibody and is non-reactive with the subject's immune systems. Examples include, but are not limited to, any of a number of standard pharmaceutical carriers such as sterile phosphate buffered saline solutions, bacteriostatic water, and the like (see, generally, Remington's Pharmaceutical Sciences 16th sup. Edition, A. Osal., Ed., 1980).

The anti-IGF-1R antibody formulations may be administered via any route capable of delivering the antibodies to the tumor site. Potentially effective routes of administration include, but are not limited to, intravenous, intraperitoneal, intramuscular, intratumor, intradermal, and the like. The preferred route of administration is by intravenous injection. A preferred formulation for intravenous injection comprises the anti-IGF-1R mAbs in a solution of preserved bacteriostatic water, sterile

unpreserved water, and/or diluted in polyvinylchloride or polyethylene bags containing 0.9% sterile Sodium Chloride for Injection, USP. The anti-IGF-1R mAb preparation may be lyophilized and stored as a sterile powder, preferably under vacuum, and then reconstituted in bacteriostatic water containing, for example, benzyl alcohol preservative, or in sterile water prior to injection.

Treatment will generally involve the repeated administration of the anti-IGF-1R antibody preparation via an acceptable route of administration such as intravenous injection (IV), at an effective dose. Dosages will depend upon various factors generally appreciated by those of skill in the art, including without limitation the type of cancer and the severity, grade, or stage of the cancer, the binding affinity and half life of the mAb or mAbs used, the degree of IGF-1R expression in the patient, the extent of circulating shed IGF-1R antigen, the desired steady-state antibody concentration level, frequency of treatment, and the influence of chemotherapeutic agents used in combination with the treatment method of the invention. Typical daily doses may range from about 0.1 to 100 mg/kg. Doses in the range of 10-500 mg mAb per week may be effective and well tolerated, although even higher weekly doses may be appropriate and/or well tolerated. The principal determining factor in defining the appropriate dose is the amount of a particular antibody necessary to be therapeutically effective in a particular context. Repeated administrations may be required in order to achieve tumor inhibition or regression. Initial loading doses may be higher. The initial loading dose may be administered as an infusion. Periodic maintenance doses may be administered similarly, provided the initial dose is well tolerated.

A patient may be diagnosed as having an IGF-1R mediated cancer e.g., where the cells overexpress IGF-1R relative to normal, in that the patient no longer responds to anti-androgen therapy and the patient diagnosed as having androgen dependent prostate cancer may be one who responds to anti-androgen therapy. The cancer will generally comprise IGF-1R-expressing cells, such that the anti-IGF-1R antibody is able to bind thereto. While the cancer may be characterized by overexpression of the IGF-1R molecule, the present application further provides a method for treating cancer which is not considered to be an IGF-1R-overexpressing cancer.

To determine IGF-1R expression in the cancer, various diagnostic assays are available. In one embodiment, IGF-1R overexpression may be analyzed by immunohistochemistry (IHC). Paraffin embedded tissue sections from a tumor biopsy may be subjected to the IHC assay and accorded a IGF-1R protein staining intensity criteria as follows:

Score 0 no staining is observed or membrane staining is observed in less than 10% of tumor cells.

Score 1+a faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane.

Score 2+a weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.

Score 3+a moderate to strong complete membrane staining is observed in more than .sup.10 % of the tumor cells.

Those tumors with 0 or 1+ scores for IGF-1R expression may be characterized as not overexpressing IGF-1R, whereas those tumors with 2+ or 3+ scores may be characterized as overexpressing IGF-1R.

Alternatively, or additionally, FISH assays such as the INFORM™ (sold by Ventana, Arizona) or PATHVISION™ (Vysis, Illinois) may be carried out on formalin-fixed, paraffin-embedded tumor tissue to determine the extent (if any) of IGF-1R overexpression in the tumor.

As understood by those of skill in the art, assay methods for identifying compounds that modulate IGF-1R activity (e.g., antagonists) generally require comparison to a control. One type of a "control" cell or "control" culture is a cell or culture that is treated substantially the same as the cell or culture exposed to the test compound, except the control culture is not exposed to test compound. Another type of "control" cell or "control" culture may be a cell or a culture of cells which are identical to the transfected cells, except the cells employed for the control culture do not express the recombinant IGF-1R expressed in the transfected cells. In this situation, the response of test cell to test compound is compared to the response (or lack of response) of receptor-negative (control) cell to test compound, when cells or cultures of each type of cell are exposed to substantially the same reaction conditions in the presence of compound being assayed.

In order to provide a basis for the diagnosis of disease associated with aberrant expression of a human IGF-1R, a "normal" or standard profile for expression is established. Standard values for IGF-1R expression can be established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with an antibody of the invention, that which recognized the same epitope on IGF-1R as F50035, under conditions favoring the formation of complex therebetween. The amount of standard complex formation may be quantified by various methods, but preferably by photometric means. Quantities of IGF-1R expressed in control and disease samples from biopsied tissues or biological samples are compared with the standard values. Deviation between standard and subject values, in turn, will establish the parameters for diagnosing the disease.

A variety of protocols for detecting and measuring the expression of IGF-1R, using either polyclonal or monoclonal antibodies specific for the protein, are known in the art. See, e.g., Hampton, R. *et al.* (1990) Serological Methods, a Laboratory Manual, APS Press, St Paul, Minn., Section IV; and Maddox, D. E. *et al.* (1983) J. Exp. Med. 158:1211-1216.

IGF-1R overexpression or amplification may be evaluated using an *in vivo* diagnostic assay, e.g. by administering a molecule (such as an antibody) which binds the molecule to be detected and is tagged with a detectable label (e.g. a radioactive isotope or a fluorescent label) and externally scanning the patient for localization of the label.

Currently, depending on the stage of the cancer, cancer treatment involves one or a combination of the following therapies: surgery to remove the cancerous tissue, radiation therapy and chemotherapy. Anti-IGF-1R antibody therapy may be especially desirable in elderly patients who do not tolerate the toxicity and side effects of chemotherapy well, in metastatic disease where radiation therapy

has limited usefulness, and for the management of a cancerous condition unresponsive to conventional protocols.

The tumor targeting and internalizing anti-IGF-1R antibodies of the invention are useful to alleviate IGF-1R-expressing cancers, e.g. prostate and bladder cancers upon initial diagnosis of the disease or during relapse.

For therapeutic applications, the anti-IGF-1R antibodies identified, for example, in a competitive format using the reference antibody – F50035 can be used alone, or in combination therapy with, e.g., hormones, prodrugs, radiolabelled compounds eyc, or with surgery, cryotherapy, and/or radiotherapy, notably for prostate cancers, also particularly where shed cells cannot be reached. Anti-IGF-1R antibody treatment can be administered in conjunction with other forms of conventional therapy, either consecutively with, pre- or post-conventional therapy. Chemotherapeutic drugs such as TAXOTERE® (docetaxel), TAXOL® (palictaxel), estramustine and mitoxantrone are used in treating metastatic and hormone refractory prostate cancer, in particular, in good risk patients.

In the present method of the invention for treating or alleviating cancer, in particular, androgen independent and/or metastatic prostate cancer, the cancer patient can be administered anti-IGF-1R antibody in conduction with treatment with the one or more of the preceding chemotherapeutic agents. In some instances, combination therapy with palictaxel and modified derivatives (see, e.g., EP0600517) is contemplated. The anti-IGF-1R antibody will be administered with a therapeutically effective dose of the chemotherapeutic agent. In another embodiment, the anti-IGF-1R antibody is administered in conjunction with chemotherapy to enhance the activity and efficacy of the chemotherapeutic agent, e.g., paclitaxel. The Physicians' Desk Reference (PDR) discloses dosages of these agents that have been used in treatment of various cancers. The dosing regimen and dosages of these aforementioned chemotherapeutic drugs that are therapeutically effective will depend on the particular cancer being treated, the extent of the disease and other factors familiar to the physician of skill in the art and can be determined by the physician.

In a particular embodiment, an immunoconjugate comprising the anti-IGF-1R antibody conjugated with a cytotoxic agent can be administered to the patient. Preferably, the immunoconjugate bound to the IGF-1R protein is internalized by the cell, resulting in increased therapeutic efficacy of the immunoconjugate in killing the cancer cell to which it binds. In a preferred embodiment, the cytotoxic agent targets or interferes with the nucleic acid in the cancer cell. Examples of such cytotoxic agents are described above and include maytansinoids, calicheamicins, ribonucleases and DNA endonucleases.

The anti-IGF-1R antibodies or immunoconjugates may be administered to a human patient, in accord with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

Other therapeutic regimens may be combined with the administration of any anti-IGF-1R

antibody of the preceding embodiments. The combined administration may further include co-administration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preferably such combined therapy results in a synergistic therapeutic effect.

It may, in certain circumstances, be desirable to combine administration of the anti-IGF-1R antibody or antibodies, with administration of an antibody directed against another tumor antigen associated with the particular cancer.

In another embodiment, the antibody therapeutic treatment method of the present invention involves the combined administration of an anti-IGF-1R antibody (or antibodies) and one or more chemotherapeutic agents or growth inhibitory agents, including co-administration of cocktails of different chemotherapeutic agents. Chemotherapeutic agents include estramustine phosphate, prednimustine, cisplatin, 5-fluorouracil, melphalan, cyclophosphamide, hydroxyurea and hydroxyureataxanes (such as paclitaxel and doxetaxel) and/or anthracycline antibiotics. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M.C. Perry, Williams & Wilkins, Baltimore, Md. (1992).

The antibody (or antibodies) identified by methods of the invention may be combined with an anti-hormonal compound; e.g., an anti-estrogen compound such as tamoxifen; an anti-progesterone such as onapristone (see, EP 616 812); or an anti-androgen such as flutamide, in dosages known for such molecules. Where the cancer to be treated is androgen independent cancer, the patient may previously have been subjected to anti-androgen therapy and, after the cancer becomes androgen independent, the anti-IGF-1R antibody (and optionally other agents as described herein) may be administered to the patient.

At times, it may be beneficial to also co-administer a cardioprotectant (to prevent or reduce myocardial dysfunction associated with the therapy) or one or more cytokines to the patient. In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy, before, simultaneously with, or post antibody therapy. Suitable dosages for any of the above co-administered agents are those presently used and may be lowered due to the combined action (synergy) of the agent and anti-IGF-1R antibody.

For the prevention or treatment of disease, the dosage and mode of administration will be chosen by the physician according to known criteria. The appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Preferably, the antibody is

administered by intravenous infusion or by subcutaneous injections. Depending on the type and severity of the disease, about 1 .mu.g/kg to about 50 mg/kg body weight (e.g. about 0.1-15 mg/kg/dose) of antibody can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A dosing regimen can comprise administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the anti-IGF-1R antibody. However, other dosage regimens may be useful. A typical daily dosage might range from about 1 .mu.g/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The progress of this therapy can be readily monitored by conventional methods and assays and based on criteria known to the physician or other persons of skill in the art.

An effective amount of an antibody or compound of the present invention to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 .mu.g/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer a molecule of the present invention until a dosage is reached that provides the required biological effect. The progress of this therapy is easily monitored by conventional assays.

Aside from administration of the antibody protein to the patient, the present application contemplates administration of the antibody by gene therapy. Such administration of nucleic acid encoding the antibody is encompassed by the expression "administering a therapeutically effective amount of an antibody". See, for example, WO96/07321 published Mar. 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

An individual suffering from an IGF-1R mediated disorder may be treated using antibodies of the present invention or compounds identified in screens using the antibodies. By treatment is meant at least an amelioration of the symptoms associated with the pathological condition afflicting the host, where amelioration is used in a broad sense to refer to at least a reduction in the magnitude of a parameter, e.g. symptom, associated with the pathological condition being treated, such as neuronal cell death. As such, treatment includes situations where the pathological condition, or at least symptoms associated therewith, are completely inhibited, e.g. prevented from happening, or stopped, e.g. terminated, such that the host no longer suffers from the pathological condition, or at least the symptoms that characterize the pathological condition.

A variety of individuals are treatable according to the subject methods. Generally such individuals are "mammals" or "mammalian," where these terms are used broadly to describe organisms which are within the class mammalia, including the orders carnivore (e.g., dogs and cats), rodentia (e.g.,

mice, guinea pigs, and rats), and primates (e.g., humans, chimpanzees, and monkeys). In many embodiments, the individuals will be humans.

In certain embodiments, the methods of treatment involve administration of an effective amount of a compound that modulates, e.g. inhibits, the interaction of IGF-1R with its cellular targets. The compound is preferably an antibody of the invention that targets the IGF-1R epitope contained within the extracellular domain of IGF-1R. In a preferred embodiment the antibodies are human or humanized, such that any undesirable immune response in the patient is minimized.

The anti-IGF-1R antibodies may be administered using any convenient protocol capable of resulting in the desired therapeutic activity. Thus, the agent can be incorporated into a variety of formulations for therapeutic administration. More particularly, the agents of the present invention can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents (Remington: The Science and Practice of Pharmacy, 19th sup. Edition, Alfonso, R., ed., Mack Publishing Co. (Easton, Pa.: 1995)), and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants and aerosols.

Anti-IGF-1R antibodies to be used for *in vivo* administration must be sterile. The sterility may be accomplished by filtration using sterile filtration membranes, prior to or following lyophilization and reconstitution. The anti-IGF-1R antibodies may be stored in lyophilized form or in solution.

The anti-IGF-1R antibody compositions may be placed into a container with a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

In pharmaceutical dosage forms, the antibodies or other compounds may be used alone or in appropriate association, as well as in combination with other pharmaceutically active or inactive compounds. The following methods and excipients are merely exemplary and are in no way limiting.

The agents can be formulated into preparations for injection by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

Administration of the agents can be achieved in various ways, including intracranial, either injected directly into the brain tissue or injected into the cerebrospinal fluid, oral, buccal, rectal, parenteral, intraperitoneal, intradermal, transdermal, intracheal, intracerebral, etc., administration. The antibodies may be administered in combination with one or more additional therapeutic agents. Administration may be chronic or intermittent, as deemed appropriate by the supervising practitioner, particularly in view of any change in the disease state or any undesirable side effects. Administration "in combination with" one or more further therapeutic agents includes both simultaneous (at the same time) and consecutive administration in any order. "Chronic" administration refers to administration of the

agent in a continuous manner while "intermittent" administration refers to treatment that is not done without interruption. In a particular embodiment, antibodies of the invention are administered by intracranial injection. The injection will typically be directly into affected brain regions or into the cerebrospinal fluid.

Articles of Manufacture and Kits

Another embodiment of the invention is an article of manufacture containing materials useful for the treatment of anti-IGF-1R expressing cancer, in particular prostate cancer and bladder cancer. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the cancer condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-IGF-1R antibody of the invention. The label or package insert indicates that the composition is used for treating prostate cancer, androgen independent prostate cancer, or androgen dependent prostate cancer, or bladder cancer. The label or package insert will further comprise instructions for administering the antibody composition to the cancer patient. Additionally, the article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

Kits are also provided that are useful for various purposes, e.g., for IGF-1R cell killing assays, for purification or immunoprecipitation of IGF-1R from cells. For isolation and purification of IGF-1R, the kit can contain an anti-IGF-1R antibody coupled to beads (e.g., sepharose beads). Kits can be provided which contain the antibodies for detection and quantitation of IGF-1R in vitro, e.g. in an ELISA or a Western blot. As with the article of manufacture, the kit comprises a container and a label or package insert on or associated with the container. The container holds a composition comprising at least one anti-IGF-1R antibody of the invention. Additional containers may be included that contain, e.g., diluents and buffers, control antibodies. The label or package insert may provide a description of the composition as well as instructions for the intended in vitro or diagnostic use.

The above and other features of the invention will now be described more particularly with reference to the accompanying figures and pointed out in the claims. The particular embodiments described below, including the experiments conducted and achieved are provided by way of illustration and are not meant to be construed as a limitation on the scope of the invention. It will be apparent to one of ordinary skill in the art that many modifications can be made to the present invention without

departing from the spirit or essential characteristics of the invention. All references cited throughout the specification, are hereby expressly incorporated by reference in their entirety.

EXAMPLE 1

1. Overall Experimental Strategy

The epitope excision method consists of several steps: (i) the mAb is immobilized on beads (typically CNBr-activated sepharose beads); (ii) The antigen in its native form is incubated with the mAb to form the immune complex; (iii) The complex is subjected to digestion with endoproteinase enzymes in carefully controlled conditions ("limited proteolysis"). While the regions of the antigen which are not in contact with the antibody generate peptide fragments that become free from the immune complex and can be washed away from the beads, the parts of the antigen which correspond to the antigenic region (i.e. the epitope) remain bound to the antibody; (iv) The bound peptides are identified and characterized by direct analysis of the beads by Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS).

In order to define the smallest region recognized by the antibody (minimal epitope) steps (iii)-(iv) can be repeated on the antigen still bound to the mAb, and after digestion with the first enzyme. Sub-digestions are performed with endoproteinases of different specificity from the one used in the first step of hydrolysis, or with amino- and/or carboxypeptidases, which digest the antigenic peptides starting from their N-or C-terminus, respectively.

2. Detailed experimental conditions and results.

<u>Immobilization of the mAb to CNBr-activated Sepharose beads</u>.

CNBr-activated Sepharose beads were resuspended in HCl 1mM at a concentration of 50mg/ml. 100 µl of beads were then packed into two 0.8 ml micro-columns and extensively washed with 20 ml each of 1mM HCl. The two columns were then equilibrated with 20 ml each of 0.1 M $NaHCO_3$, 0.5 M NaCl at pH=8.3 (equilibration buffer). One column was subjected to the coupling reaction with the antibody in the following conditions: 93 µl of equilibration buffer + 7 µl of F50035 (50µl g, Lot #75667-62RTW). The other column was incubated in the presence of the buffer but without antibody to serve as a blank in the following conditions: 93µl of equilibration buffer + 7µl of (PBS buffer pH=6.5).

The coupling reaction was performed at 37°C for two hours with gentle shaking. After coupling the columns were washed with 20 ml each of 0.1 M Tris-HCl, 0.5 M NaCl at pH=8.0, and then incubated in the same buffer at 37°C for two hours, to achieve complete deactivation of all the unreacted sites still present on the sepharose beads. The beads were then washed with 20 ml of PBS buffer pH=7.4

<u>Formation of the immune complex</u>.

The beads with bound F50035 were incubated with extracellular domain (ECD) of IGF-1R (R&D Systems, Catalog Number 391-GR) at a concentration of 0.5 µl g/µl in 100µl of PBS pH=7.4, for 2 h at 37°C, with gentle shaking. The beads were then washed with 20 ml of PBS. An aliquot of sample and control beads was deposited onto the MALDI target plate and subjected to direct MALDI-MS analysis in the presence of sinapinic acid (SA) as matrix. Referring to Figure 1, the data show that the antigen specifically bound to the mAb-immobilized beads.

Limited proteolysis with Endoproteinase ArgC.

In order to avoid excess hydrolysis of the antigen and possible los of the epitope, the amount of proteolytic enzyme normally used in epitope excision experiments was reduced considerably.

The immune complex, *supra*, was incubated overnight in the presence of Endoproteinase ArgC, a known reagent that specifically cleaves C-terminally to Arg residues, at a concentration of 0.5 µg per 100µl of mAb -conjugated beads. Since the MALDI-MS analysis of the beads revealed that the antigen was only partially hydrolized, the immune complex was incubated again in the presence of 1µl g Arg C for 16 h. The latter treatment was repeated twice. Only after the third step of hydrolysis MALDI-MS analysis of the beads revealed full digestion of the antigen. The above experiment resulted in various peptide fragments at different m/z, e.g., 14979.62, 13442.98, 12187.96 and 4790.68. Importantly, these fragments were not detected in the blank. The data is shown in Figure 2. Enzymatic digestion in solution of the F50035 mAb showed that the species at m/z 13442.98 and 12187.96 originated from either the antibody or the protease used in the experiments, leaving as epitope-specific peptides the two species at m/z 14979.62 and 4790.68.

The species at m/z 14979.62 (Species A fragment) was characterized as the disulfide-linked homodimer of peptide 642-707, at the C-terminus of the α-chain. Refer to Figure 3. This peptide contains four cysteine residues at amino acid positions 662, 669, 670 and 672 with residues at amino acid positions 669, 670 and 672 (in double underline) involved in three disulfides linking the two α-chain monomers. The expected molecular weight of the homodimer (assuming the presence of the three disulfide bonds) is 14975.74 Da. The species at m/z. 4790.68 (Species B fragment) was tentatively assigned to the N-terminal peptide 1-41 of the α-chain, which contains two cysteine residues (C_3 and C_{22}, double underline) involved in a disulfide bond (expected $MW_{oxidized}$ 4792.50). By these assignments, the complex was observed to protect R_{10} and R_{18} in the N-terminal peptide and R_{689} in the C-terminal homodimer (Figure 3, broken underline).

On-target reduction experiments of the mixture resulting from ArgC digestion revealed the disappearance of all the high MW signals, with formation of two major species at m/z 7492.91 and 6519.79. The first one corresponds to the reduced peptide defined by amino acid positions 642-707 (expected $MW_{reduced}$ 7491.37), thus confirming the initial attribution, while the second one corresponds to peptide 1-59 in the F50035 Vk chain (expected $MW_{reduced}$ 6517.42). No signal was detected for the reduced peptide 1-41 possibly due to low sensitivity.

Sub-digestions with endoproteinase GluC and carboxypeptidase.

The observed degree of agreement between the expected and measured mass values was within the norm considering that the mass calibration was conducted with an external standard. To further corroborate the results, the beads were split into three different aliquots and the ArgC-derived peptides, still bound to the mAb, were subjected to independent incubations in the presence of endoprotease GluC and carboxypeptidase. The resulting data not only corroborated the mass spec. data but, in addition, it allowed for the determination of the minimal epitope.

Sub-digestion of the F50035/IGF-1R immune complex with Endoproteinase GluC.

The beads were incubated overnight in the presence of endoproteinase GluC (1µl g/100µl of beads) and then subjected to MALDI-MS analysis. This second step of digestion led to the formation of several species as detailed in Figure 4, and summarized in Tables 1-2:

Table 2. Peptides originating from the species at m/z 14979.62 upon incubation with GluC

Measured m/z	Expected $MW_{oxidized}$	Peptide	Cleaved AA
12501.40	12505.12	(653-707) + (653-707)	E_{652} (both peptide chains)
10701.33	10707.18	(661-707) + (661-707)	E_{660} (both peptide chains)
2549.09	2549.93	(688-707)	E_{687}

Table 3. Peptides originated from the species at m/z 4790.68 incubated with GluC

Measured m/z	Expected $MW_{oxidized}$	Peptide	Cleaved AA
1792.27	1792.08	(27-41)	E_{26}

Sub-digestion of the F50035/IGF-1R immune complex with Carboxypeptidase A.

The beads (100 µl) were incubated for 1 hour in the presence of 1µl g/µl of carboxypeptidease A (CPA), and the results are summarized in Tables 3-4:

Table 4. Peptides originated from the species at m/z 14979.62 upon incubation with CPA

Measured m/z	Expected $MW_{oxidized}$	Peptide	Removed AA
14681.87	14690.44	(642-705) + (642-707)	$E_{706}R_{707}$ on one peptide chain
14391.53	14405.14	(642-705) + (642-705)	$E_{706}R_{707}$ on both peptide chains

Table 5. Peptides originated from the species at m/z 4791.84 upon incubation with CPA

Measured m/z	Expected $MW_{oxidized}$	Peptide	Removed AA
4476.21	4475.14	(1-39)	$Y_{40}R_{41}$

Referring to Figure 3, increasing the amount of CPA (from 1 to 2.5 µl g/µl) or the incubation time (from 1 hour to overnight) did not lead to further removal of amino acids from both species. E_{706} and Y_{40} (triple underline), which were removed by CPA, are likely not included in the epitope region.

3. Conclusions

In summary, mAb F50035 recognizes a discontinuous epitope including both the N-terminal and the C-terminal sequence of the α-chain. Referring to figure 5, the minimal identified epitope includes the N-terminal region G_{27}-R_{41} and the C-terminal region Y_{688}-R_{707} - Figure 5, boxed area.

The location of the epitope deriving from the above experiments is entirely consistent with the available information on the IGF-1/IGF-1R complex: (a) the MS-mapped epitope includes most of the amino acid residues which are known to be critical for binding of IGF-1 (cyan boxes); in particular, it includes the C-terminal sequence 692-702, which is the most important region for hormone binding, as established by alanine-scanning mutagenesis (Whittaker *et al.*, *J. Biol. Chem.* 276 (2001) 43980); (b) Accordingly, mAb F50035 inhibits binding of IGF-1 to IGF-1R; (c) The recent 3D reconstruction of the (highly similar) insulin receptor by scanning transmission electron micrographs (STEM) positions the N-terminal and the C-terminal region of the α-chain close to each other (Yip& Ottensmeyer, *J. Biol. Chem.* 278 (2003) 27329).

Referring to Figure 6, it is evident that the sequence of the two regions of the IGF-1R which are recognized by F50035 (IGF-1R epitope) is highly conserved among human, rat and mouse epitope.

EXAMPLE 2 - Western blot experiments

Material and Methods

Electrophoresis and western blot

Recombinant human insulin-like growth factor 1 receptor extracellular domain (IGF-1R ECD, R&D Systems, Lille, France) was submitted to SDS-PAGE electrophoresis on 8% homogeneous polyacrylamide gels (Invitrogen, Cergy Pontoise, France) under reducing and non-reducing conditions. Proteins were further transferred onto nitrocellulose membrane. After blocking with 1% fat free milk in Tris buffered saline containing 0.1% Tween 20 overnight at room temperature, membranes were probed with antibodies 7C10 and 13F5 (at 4 ng/ml and 0.4 µg/ml in blocking buffer, respectively) for 1h at room temperature. Proteins were further detected by chemiluminescence (ECL, Amersham Biosciences, Orsay,

France) after incubation with a horseradish peroxidase-conjugated anti-mouse IgG polyclonal antibody (Amersham Biosciences) for 1 h at room temperature and extensive washes.

Results

As shown in Figure 7, the Western blot data confirms binding of the monoclonal antibodies 7C10 and 13F5 to the native $\gamma_2\beta_2$ tetrameric form of recombinant IGF-1R ECD. As well, the lack of reactivity of both 7C10 and 13F5 observed with the fully reduced form of IGF-1R suggests that the epitope of these antibodies is conformational and not linear.

EXAMPLE 3 - [^{125}I]-IGF-1 binding inhibition experiments

Material and Methods

Proteins and membrane extract

Human recombinant [^{125}I]-IGF-1 (specific activity: 2,500 Ci/mmole) was purchased from Perkin Elmer (Boston, MA, USA). Non radiolabeled recombinant human IGF-1, IGF-2 and insulin were from Sigma (Saint Quentin Fallavier, France). The anti-hIGF-1R monoclonal antibody 17-69 was from Neomarkers (Fremont, CA, USA).

Membrane extracts of NIH 3T3 cells overexpressing IGF-1R were obtained as followed. After cell lysis in 10 mM Tris-HCl pH 7.5 buffer, whole cell membranes were collected by centrifugation at 105,000 g for 1 h at 4°C. The pellet was resuspended in 50 mM Tris-HCl pH 7.5 buffer containing 150 mM NaCl, 0.5% IGEPAL, 0.5% Triton X-100, 0.25% sodium deoxycholate and protease inhibitors, and stirred overnight at +4°C. Insoluble material was separated from the soluble extract containing hIGF-1R by centrifugation at 10,000 g for 10 min at +4°C. Soluble membrane extracts were analysed for protein concentration by the bicinchoninic assay.

[^{125}I]-IGF-1 binding assay

mAb 17-69 was first coated on Protein A FlashPlate® 96-well microplates (Perkin Elmer). Two thousand µl of a 20 µg/ml mAb solution in PBS were added to each well and incubated overnight at +4°C. The buffer containing residual mAb 17-69 not attached to protein A was removed by aspiration. Two hundred µl of the membrane lysate at 100 µg/ml were further added and incubated for 2 h at room temperature to immobilize IGF-1R. Non captured proteins were removed by aspiration. Binding of [^{125}I]-IGF-1 at 100 pM to immobilized IGF-1R was measured as a function of time in the presence of the anti-hIGF-1R monoclonal antibodies 7C10 and 13F5, the monoclonal antibody 9G4 which specifically recognizes an *E. coli* protein or non-radiolabeled IGF-1 at the concentration of 1 µM in binding buffer containing 50 mM Hepes pH 7.6, 150 mM NaCl, 0.05 % Tween 20, 1 % bovine serum albumin and 1 mM PMSF. The plates were incubated at room temperature, then counted at different times on a Packard Top Count Microplate Scintillation Counter.

Results

Dose response studies with 7C10 and 13F5, such as inhibition of *in vitro* cell proliferation, suggest that these antibodies are non-competitive antagonists of IGF-1 for IGF-1R since they are able to depress IGF-1 concentration response curves. Kinetic inhibition studies were conducted to confirm the above hypothesis as well as determine the basis of the non-competitive antagonism, e.g., functional or allosteric. Towards this end, binding of [^{125}I]-IGF-1 to immobilized IGF-1R was measured over time upon addition of unlabeled IGF-1, 7C10 and 13F5. Percent of initial (zero time) [^{125}I]-IGF-1 binding was plotted as a function of time of incubation with the different ligands tested.

When compared to the dissociation curve observed for unlabeled IGF-1, the curves obtained for 7C10 and 13F5 were highly similar (Fig. 8A and B). The time observed to obtain 50% dissociation of [^{125}I]-IGF-1 bound to immobilized IGF-1R in the presence of unlabeled IGF-1 was comprised between 20 to 35 min. In contrast, 50% of [^{125}I]-IGF-1 bound dissociated after around 7 min in the presence of antibodies 7C10 and 13F5. As expected, the monoclonal antibody 9G4 was unable to dissociate [^{125}I]-IGF-1 from IGF-1R. The [^{125}I]-IGF-1 dissociation acceleration observed with 7C10 and 13F5 confirmed that the antibodies are non-competitive antagonists but also that the basis of the non-competitive antagonism is allosteric. Thus, the data suggest that 7C10 and 13F5 interact at an allosteric site of IGF-1R, distinct from the IGF-1 binding site. This interaction is hypothesized to induce a conformational change in the structure of the receptor thereby compromising its interaction with IGF-1.

EXAMPLE 4

Impact of recombinant human Insulin-Like Growth Factor-1 receptor extracellular domain (IGF-1R ECD) N-glycosylation on its recognition by A2CHM and 13F5 anti-IGF-1R monoclonal antibodies.

Material and Methods

IGF-1R ECD *N-deglycosylation* (PNGase F Kit, BioLabs Inc, New England USA)

20 µg of IGF-1R ECD (R&D Systems, Lille, France) was first denatured using 1X glycoprotein denaturing buffer (5% SDS, 10% β-mercaptoethanol) at room temperature for 10 min. The denatured receptor was then submitted to N-deglycosylation using peptide N-Glycosidase F at 37°C for 1 hour after addition of 1/10 volume 0.5M sodium phosphate pH 7.5 of buffer, and 10%NP-40. PNGase F is an amidase which hydrolyzes nearly all types of N-glycan chains from glycopeptides/glycoproteins.

Electrophoresis and western blot

IGF-1R ECD, N-deglycosylated or not was submitted to SDS-PAGE electrophoresis on 4-12% NuPage Bis-Tris gels (Invitrogen, Cergy Pontoise, France) under mild-reducing conditions (in the presence of β-mercaptoethanol without heating). Proteins were further transferred onto Hybond ECL membranes. After blocking with 1% fat free milk in Tris buffered saline containing 0.1% Tween 20 overnight at room temperature, membranes were probed with antibodies A2CHM and 13F5 (at 50 ng/ml

and 1.4 μg/ml in blocking buffer, respectively) overnight at 4°C. Proteins were further detected by chemiluminescence (ECL, Amersham Biosciences, Orsay, France) after incubation with a horseradish peroxidase-conjugated- anti-mouse IgG polyclonal antibody (for 13F5) or anti-human IgG polyclonal antibody (for A2CHM) (Amersham Biosciences) for 1 h at room temperature and extensive washes.

Results and conclusion

The monoclonal antibodies A2CHM and 13F5 were able to detect the α subunit of recombinant IGF-1R ECD (two bands at 220 000 Da and 150 000 Da) by western blot after SDS-PAGE analysis under mild-reducing conditions (β-mercaptoethanol without heating) (Figures 9A and 9B). Moreover, both A2CHM and 13F5 were also able to recognized the N-deglycosylated form of IGF-1R ECD (two bands at 150 000 Da and 100 000 Da) under mild-reducing conditions, thus corroborating the observation that N-glycosylation has no impact on the recognition of this receptor by A2CHM and 13F5 monoclonal antibodies. See Figures 9A & 9B.

EXAMPLE 5

Alanine scanning mutagenesis of the IGF-1R epitope.

To confirm that the epitope in the IGF-1R Extra Cellular Domain (ECD) recognized by the reference antibody maps in the two regions identified by LP-MS, an extensive mutagenesis study was performed by single alanine substitutions of each of the 13 amino acids in the N-terminal part of the epitope and of each of the 20 amino acids in the C-terminal of the epitope. In addition, the inventors generated 3 Insulin Receptor (IR) swap mutants, one exchanging the entire 13-amino acid N-terminal part with the corresponding region of IR, and two substituting the 20 amino acid C-terminal part with the corresponding region in the IR-A and IR-B variants of the IR.

Each of the IGF-1R mutants was transiently transfected into mouse cells that do not express IGF-1R, and tested for its ability to bind the reference antibody in a whole cell binding assay and FACS analysis. Binding capacity of the control mAb to each mutant was compared to the binding to wild-type receptor and normalized for expression in the transfected cells.

Materials and Methods

33 Ala-scan mutants of IGF-1R and the 3 IR-swap mutants (IR A and B variants for the C-terminal epitope) were obtained Geneart. All the mutant receptors were cloned into the pCMVSport5 mammalian expression vector. Each of these mutants had been individually transfected into the mouse neuronal cell line N2A by Lipofectamine 2000 reagent in accordance with the instructions from the vendor- Invitrogen.

48 hours after transfection the cells have been detached, counted and separated in two aliquots: one was used to prepare total cell lysates in 50mM Tris HCl pH 8, 150mM NaCl, 1% Triton, while the remaining cells were used for a whole cell binding assay with the reference antibody.

Cells were incubated with increasing concentrations of the reference antibody: 0.02, 0.2 and 2ug/ml, in PBS 0.2% BSA, 10nM Hepes (FACS buffer) one hour at room temperature. As a secondary reagent an anti-human total IgG, Alexa Fluor 488-conjugated (Molecular Probes) was used at the final concentration of 100ng/ml. After washing, the cells were analyzed with a Becton Dickinson FACS Calibur and the acquired data analyzed using CellQuest software (BD). Binding of control mAb to each mutant was measured calculating the Mean Fluorescence Intensity (MFI) of the transfected cells for each mAb concentration and substracted of the background MFI measured with a human isotypic IgG control at the same concentrations. Binding capacity was expressed relative to the wild type receptor.

To normalize for the expression of the transfected receptor, total cell lysates from each transfection were separated by SDS-PAGE and transferred to nitrocellulose filters. Filters were hybridized with an anti-IGF-1R ECD goat polyclonal antibody (R&D Systems). A secondary HRP-conjugated anti-goat antibody was used to reveal the Western Blot by ECL and the band corresponding to IGF-1R was quantified by a densitometer. Protein levels of each mutant were expressed as relative to the wild type IGF-1R expression.

Results

Binding analyses of the reference antibody to the Alanine scanning receptor mutants in the regions encompassing the mapped epitopes are shown in Fig 10a and 10b.

Referring to Fig.10a, shown therein is the binding capacity of each Ala mutant in the N-terminus region of the IGF-1R receptor. Data is expressed as percentage respect to the binding of the wild typeIGF-1R.

The data show that replacement of Tyrosine in position 28, Leucine in position 29, Isoleucine in position 31 and Aspartic acid in position 39 strongly reduced the binding capacity of the antibody to the receptor, confirming the critical importance of this region for binding to the control mAb.

On the other hand, as detailed in Fig. 10b, none of the single Ala mutations in the C-terminal region of the receptor produced a significant reduction in reference antibody binding affinity. Likewise, none of the three Insulin Receptor (IR) swap mutants, reduced reference antibody binding capacity. These swap mutants include the one wherein exchanging the entire N-terminal 13 amino acid region, suggest that a block substitution between IGF-1R and IR can be tolerated, while a single-point mutation can disrupt binding. In summary, the mutational study confirms that the N-terminal region of the IGF1-R receptor encompassing amino acids 28-41 is critical for reference antibody binding, while the importance of the C-terminal region could not be confirmed by this analysis.

EXAMPLE 6

Evaluation of binding affinity between mini-IGF-1R and truncated IGF-1R (1-458AA) with reference antibody (reference antibody) and commercial IGF-1R antibody (R&D Systems) using titration ELISA

Methods:

Antibodies were coated on Nunc-Maxisorp plates at concentration of 10ug/ml for reference antibody and 4ug/ml for commercial antibody. Purified IGF-1R proteins were added at starting concentration of 630pM (100ul per well) and a series of 2-fold dilution made cross the 12 column. The bound IGF-1R was detected with secondary detection antibody with HRP conjugation.
Each data point was average of two replications and standard deviations are plotted in the graph.

Results:

Referring to Figure 11, the data show that the truncated 1-458 AA receptor was able to bind to the reference antibody (F50035) with a much lower affinity than the mini-IGF-1R wherein the c-terminus comprising amino acids from position 688-706 (688-706AA) is fused with the 1-458 truncated version.

Labels for the graph Figure 11: IGF-1R mini refers to a mini IGF-1R receptor (reference antibody) assayed with commercial antibody coated on a plate; IGF-1R (1-458AA) refers to the truncated IGF-1R receptor assayed with commercial antibody coated on plate; IGF-1R (1-458AA) (reference antibody) refers to the truncated receptor assayed with the reference antibody coated on plate.

EXAMPLE 7

Experimental methods detailing the cloning, expression and characterization studies pertaining to IGF-1R.

I. Construction of IGF1R expression vectors:

The mini-IGF-1R moiety is a fusion protein comprising the amino acid sequences corresponding to or having amino acids residing at residues 1-458 and 691-707 of the alpha subunit of IGF-1R extracellular domain. Truncated IGF-1R, on the other hand, contains only the first 458 amino acids e.g., the 1-458 amino acid sequences. Refer to Figures 12 A - C. Both the mini-IGF-1R and the truncated IGF-1R(1-458) receptor proteins have Flag tag at the C-terminus of the proteins, which was used for protein purification. The DNA sequences coding for the proteins were cloned using PCR amplifications. PCR primers used to clone the proteins is detailed in Figures 12D-12F. These PCR primers were designed based on the published human IGF1R sequences, see Ullrich, A *et al.*, EMBO J. 5: 2503-2512 (1986),
and both cloning sites (*BglII* and *KpnI*) and fusion tags were introduced through PCR primers. The DNA template was supplied by an investigator resident at Merck & Co, Inc. Both DNA

sequences were amplified at 56°C annealing for 30 PCR cycles. After PCR, DNA fragments were cleaned and digested with restriction enzymes, *BglII* and *KpnI*, then cloned into pV1-JNS vector, whose general structure is detailed in Figure 12G.

II. Expression of mini-IGF-1R and truncated IGF-1R proteins in FreeStyle™ 293 cells

General methodology

A. Thawing and establishing cells

Removed cryovial containing HEK293 free style cells (Invitrogen) from liquid nitrogen and quickly thawed in 37°C water bath. After cells were thawed, cells were transferred to a shaker flask containing completed FreeStyle 293 expression media (Invitrogen)t followed by incubating cells in 37°C incubator with humidified 8% CO_2 atmosphere on orbital shaker rotating at 125 RPM.

B. Passaging cells

The cells were sub-cultured when the density of the cells approached approximately 1-3X10^6 viable cells/ml, which typically occurs every 3 to 4 days. To successfully recover cells, it is preferable for te cell to be subcultured for two passages prior to use in transfection.

C. Cell viability determination

Cell viability was determined with trypan blue viability test as following:

1. Transfer 0.9 ml of 0.4% Trypan Blue (Invitrogen) + 0.1 ml of cell suspension in an Eppendorf Tube.
2. Gently mix to avoid cell clusters by pipetting up and down.
3. Allow to stand for 2 minutes (viable cells will take up dye if incubation goes too long)
4. Pipet enough of the mixture into cover slipped chambers of hemacytometer.
5. Count viable and non-viable cells in 5 squares and cell density at 20-50 cells per square.

D. Transfection

Before transfecting cells, cell density and viability were determined using trypan blue exclusion method as described above. Cells were collected in an appropriate vessel and centrifuged at 1000 RPM for 5 minutes. Then the cells were re-suspended in fresh FreeStyle 293 Expression Media by gentle pippeting to cell density of 1x10^6 cells/ml. For 1 liter of transfection

culture, 1mg DNA were added into a total of 32.5ml of Opti-MEM I (Invitrogen) and mixed gently. Then 1.5ml of 293fectin^{TM} was diluted in a separate tube containing 32.5 ml of Opti-MEM I and mixed gently. Both solutions were incubated separately at room temperature for 5 minutes. After the incubation, the two solutions were mixed together for a total volume of 65ml. The mixture was incubated at room temperature for 30 minutes. Then the DNA/293fectin^{TM}/Opti-MEM solution was added to the cell culture flask, followed by swirling the flask to mix. The transfected cell culture was placed in 37°C, 8% CO2 incubator with shaking at 125 RPM. The expression culture was collected after 5 days.

III. Purification of IGF-1R proteins from cell culture

After removing cells by centrifugation, the culture supernatants were adjusted to 1x Tris buffered saline solution (TBS, 50mM tris, pH7.4, 150mM NaCl) by adding10x concentrated TBS. Anti-Flag affinity resin (Sigma) was prepared by washing with 5 volume of 1x TBS. Samples were loaded onto the column by gravity and the column was washed with 5 volumes of 1X TBS. Elution of IGF1R proteins were completed with 50mM citrate buffer, pH3.5, and the pH of the elution was adjusted with 1M Tris, pH8.0, immediately following elution. Protein concentrations were determined using 280nm absorbance and purity was checked on SDS-PAGE. Refer to Figure 12H.

IV. Binding of Mini-IGF1R and MK-0646 in ELISA

Reference antibody (F50035) was coated on Nunc-Maxisorp plates at concentration of 10ug/ml. Purified IGF-1R proteins were added at starting concentration of 630pM (100ul per well) and a series of 2-fold dilution made cross the 12 columns. The bound IGF-1R was detected with secondary detection antibody with HRP conjugation (R&D Systems). Each data point was average of two replications and standard deviations are plotted in the graph. The assay results shown in 12I indicate that the truncated IGF-1R (1-458 AA) bound to the reference antibody with an affinity lower than that of the mini-IGF-1R moiety (fusion protein comprising the c-terminus (688-706AA) fused to the 1-458 truncated version. The complete IGF1R extracellular domain protein (R&D System) showed the highest binding among the three receptors.

V. Biacore analysis of the binding constants

The reference antibody sample (lot 0646HSS001A001) was serially diluted from 800nM-0.625nM in HBS buffer and injected over a CM5 sensor chip coupled with 100RU IGF-IR, truncated IGF-IR and mini-IGF-IR. Each receptor was chemically linked on to a separate flow cell on the sensor chip. Kinetics evaluation was performed by the Biaevaluation software based on a Langmuir 1:1 fit.

The pooled SA material was injected into all of the flow cells. A blank flow cell was used to subtract out any non-specific binding of the antibody to the sensor chip. While the reference antibody sample appeared to bind to all of the receptors in a similar fashion, as evidenced by the Rmax, most of the receptor was did not bind the antibody . Rmax refers to the maximum binding capacity where all of the ligand binding sites are bound by the analyte. Typically Rmax values should be between 100-150RU. . When immobilizing the receptors onto the sensor chips, an RU level is targeted but the actual amount bound can vary from chip to chip. The antibody appears to associate and dissociate from each of the receptors in a similar fashion, there does not appear to be a difference in the K_D values for each receptor. Results showing Rmax values less than above range is not fatal and may suggest that the receptor is not completely bound by the antibodies. This may be due to various factors including variations in optimizing the experimental design etc. Results are summarized in the table below.

Table 6

Receptor	k_a (1/Ms)	k_d (1/s)	K_A (1/M)	K_D (M)	Rmax
IGF-IR	2.55E5	3.08E-4	8.30E8	1.20E-9	74.0
Truncated IGF-IR	2.04E5	3.48E-4	5.86E8	1.71E-9	73.4
Mini-IGF-IR	2.14E5	2.95E-4	7.26E8	1.38E-9	63.9

Example 8

MATERIALS AND METHODS

Equipment and materials

BIAcore T100 instrument, CM5 biosensor chips, HBS-EP buffer, acetate buffer pH 5, Glycine-HCl buffer pH 1.5, amine coupling kit were from BIAcore (Upsala, Sweden). Anti-human IgG Fc was from Jackson ImmunoResearch Laboratories Inc. (West Grove, USA), soluble human insulin-like growth factor-1 (hIGF1-R) extra-cellular domain (ECD) was from R&D Systems (Minneapolis, USA), mini-IGF1R and mini-IGF1R (truncated) were from Merck.

Biacore Assays

All experiments were performed at 25°C at a flow rate of 40 µl/min. To prepare a BIAcore assay (see Figure 13), an anti-human IgG-Fc antibody (50 µg/ml each in acetate buffer, pH 5.0) was immobilized onto a carboxymethyl dextran sensorchip (CM5) using amine coupling procedures as described by the manufacturer. 10000 resonance units (RU) of anti-IgG Fc antibodies were linked respectively onto

Flowcells (FC) 1 and 2. Purified Mabs to be tested were diluted at a concentration of 5 µg/ml in 0.5% P20, HBS-EP buffer and injected on FC2 to reach 500 to 1000 RU. FC1 was used as the reference cell. Specific signals correspond to the difference of signals obtained on FC2 *versus* FC1. The analyte (soluble hIGF1-R, mini-IGF1R, or mini-IGF1R (truncated form)), was injected during 90 sec at five different concentrations (100, 50, 25, 12.5 and 6.25 nM) in 0.5 % P20, HBS-EP buffer. These concentrations were prepared from stock solution in 0.5 % P20, HBS-EP. The dissociation phase of the analyte was monitored over a 10 minutes period. Running buffer was also injected under the same conditions as a double reference. After each cycle (antibody + analyte injection), both Flowcells were regenerated by injecting 20 to 45 µl of Glycine-HCl buffer pH 1.5. This regeneration is sufficient to eliminate all Mabs and Mab/analyte complexes captured on the sensorchip.

Result

The sensorgram corresponding to the different analyte concentrations is represented in Figure 13 which details a human IgG1 Mab directed against the constant Fc portion, which was covalently attached onto a CM5 sensor surface. A limited amount of Mab to be tested was immobilized and used to capture the analyte..

The binding of the anti-IGF-1R Mab (reference antibody) to the analytes (hIGF-1R-ECD, mini-IGF1R, or mini-IGF1R (truncated form) was characterized by the association and dissociation rate constants k_a and k_d, respectively (Table I). The equilibrium dissociation constant (KD) was calculated by the ratio between dissociation (koff) and association (kon) rate constants. The sensorgram corresponding to the different analyte concentrations is represented in the Figure 14.

Table 7. KD of the hIGF-1R mAb (Reference Antibody) reacting to different IGF-1R antigens

MK-0646 Mab / Antigen	Kon	Koff	KD
MK-0646 Mab / mini-IGF-1R	3.96E+04	2.66E-04	6.70E-09
MK-0646 Mab/ mini-IGF-1R (truncated)	3.77E+04	3.90E-04	1.04E-08
MK-0646 Mab / IGF-1R (R&D)	2.77E+05	1.40E-04	5.04E-10

WHAT IS CLAIMED:

1. A recombinant cell that expresses a chimeric monoclonal antibody or antigen-binding fragment thereof that binds to the same conformational epitope as that bound by humanized monoclonal antibody F50035 or a fragment thereof.

2. An IGF-1 receptor antagonist which specifically binds to a insulin-like growth factor 1 receptor (IGF-1R), wherein said antagonist or a fragment thereof binds to an epitope to which monoclonal antibody F50035 binds.

3. A nucleic acid molecule comprising a sequence of nucleotides that encode a conformational epitope comprising a primary sequence of amino acids at positions 27-41 and 688 through 707, wherein the amino acids at positions 27-41 are GYHILLISKAEDYR (SEQ ID NO:1) and said amino acids at positions 688-707 are YRKVFENFLHNSIFVPRPER (SEQ ID NO:2).

4. An isolated monoclonal antibody that specifically binds a conformational epitope expressed by an extracellular domain of native IGF-1R, wherein said epitope comprises a primary sequence of amino acids at positions 27-41 and 688 through 707, and wherein the amino acids at positions 27-41 are GYHILLISKAEDYR (SEQ ID NO:1) and said amino acids at positions 688-707 are YRKVFENFLHNSIFVPRPER (SEQ ID NO:2) and wherein the antibody is capable of inhibiting binding of a native ligand to said IGF-1R.

5. An isolated monoclonal antibody or a functional fragment thereof that specifically binds to the same conformational epitope as that bound by human monoclonal antibody F50035.

6. The antibody according to claim 5, wherein said antibody is a chimeric antibody.

7. The antibody according to claim 5, wherein said antibody is a humanized antibody.

8. The antibody according to claim 5, wherein said antibody is a fully human antibody.

9. The monoclonal antibody of claim 5, wherein said monoclonal antibody is a single-chain variant fragment.

10. The monoclonal antibody of claim 5, in association with a therapeutically acceptable carrier.

11. A method of producing the antibody or a fragment thereof of claim 4, comprising

a) introducing an immunogenic epitope of a receptor protein wherein said conformation immunogenic epitope comprises the primary sequence of amino acids as set forth in claim 3, into an animal, and

b) recovering said antibody of fragment thereof.

12. A method for treatment of an IGF-1R mediated disorder, comprising administering to a patient an effective amount of a monoclonal antibody of claim 4.

13. The method of claim 12, wherein the patient is a mammalian patient.

14. The method of claim 12, wherein the mammalian patient is human.

15. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the antibody of claim 4.

16. A vector comprising the nucleic acid molecule of claim 15.

17. A monoclonal antibody specifically immunoreactive with a receptor protein carrying a conformational epitope for use in the detection of human prostate, breast, colon cancer, wherein said epitope comprises a primary sequence of amino acids at positions 27-41 and 688 through 707, and wherein the amino acids at positions 27-41 are GYHILLISKAEDYR (SEQ ID NO:1) and said amino acids at positions 688-707 are YRKVFENFLHNSIFVPRPER (SEQ ID NO:2).

18. A monoclonal antibody specifically immunoreactive with a polypeptide carrying an epitope recognized by F50035.

19. A hybridoma cell line capable of producing the antibody of claim 4, specific for the polypeptide carrying a conformational epitope comprising a primary sequence of amino acids at positions 27-41 and 688 through 707, and wherein the amino acids at positions 27-41 are GYHILLISKAEDYR (SEQ ID NO:1) and said amino acids at positions 688-707 are YRKVFENFLHNSIFVPRPER (SEQ ID NO:2).

20. An immunoassay method utilizing the monoclonal antibody according to claim 4, to assay for test material comprising a protein carrying a conformational epitope comprising a primary sequence of amino acids at positions 27-41 and 688 through 707, and wherein the amino acids at positions 27-41 are GYHILLISKAEDYR (SEQ ID NO:1) and said amino acids at positions 688-707 are YRKVFENFLHNSIFVPRPER (SEQ ID NO:2), comprising the steps of: contacting a test material comprising a protein carrying said epitope with said monoclonal antibody; and determining the presence of said epitope in the test material.

21. A monoclonal antibody specifically immunoreactive with the a protein carrying a conformational epitope comprising a primary sequence of amino acids at positions 27-41 and 688 through 707, and wherein the amino acids at positions 27-41 are GYHILLISKAEDYR (SEQ ID NO:1) and said amino acids at positions 688-707 are YRKVFENFLHNSIFVPRPER (SEQ ID NO:2) for use in delivering therapeutic agents to tumor cells.

22. A monoclonal antibody specifically immunoreactive with the a protein carrying a conformational epitope comprising a primary sequence of amino acids at positions 27-41 and 688 through 707, and wherein the amino acids at positions 27-41 are GYHILLISKAEDYR (SEQ ID NO:1) and said amino acids at positions 688-707 are YRKVFENFLHNSIFVPRPER (SEQ ID NO:2) for use in directing imaging agents to tumor cells.

23. A monoclonal antibody specifically immunoreactive with the a protein carrying a conformational epitope comprising a primary sequence of amino acids at positions 27-41 and 688 through 707, and wherein the amino acids at positions 27-41 are GYHILLISKAEDYR (SEQ ID NO:1) and said amino acids at positions 688-707 are YRKVFENFLHNSIFVPRPER (SEQ ID NO:2) for use in detecting said epitope in paraffin fixed secretions of human tissues.

24. An IGF-1 receptor antagonist which specifically binds to a IGF-1 receptor, wherein said antagonist or a fragment thereof binds to an epitope to which monoclonal antibody F50035 binds.

25. An IGF-1R epitope agonist comprising an IGF-1R epitope.

26. An antibody that specifically binds mature human IGF-1R wherein the antibody binds the same epitope on mature human IGF-1R as monoclonal antibody F50035, humanized antibody F50035 or a functionally equivalent fragment thereof.

27. An antibody that specifically binds mature human IGF-1R wherein the antibody binds the same epitope on mature human IGF-1R as chimeric monoclonal antibody F50035 or humanized

antibody F50035.

28. The antibody of claim 27 that specifically binds mature human IGF-1R with an affinity constant that is within ten-fold the affinity constant of monoclonal antibody F50035 for human IGF-1R.

5

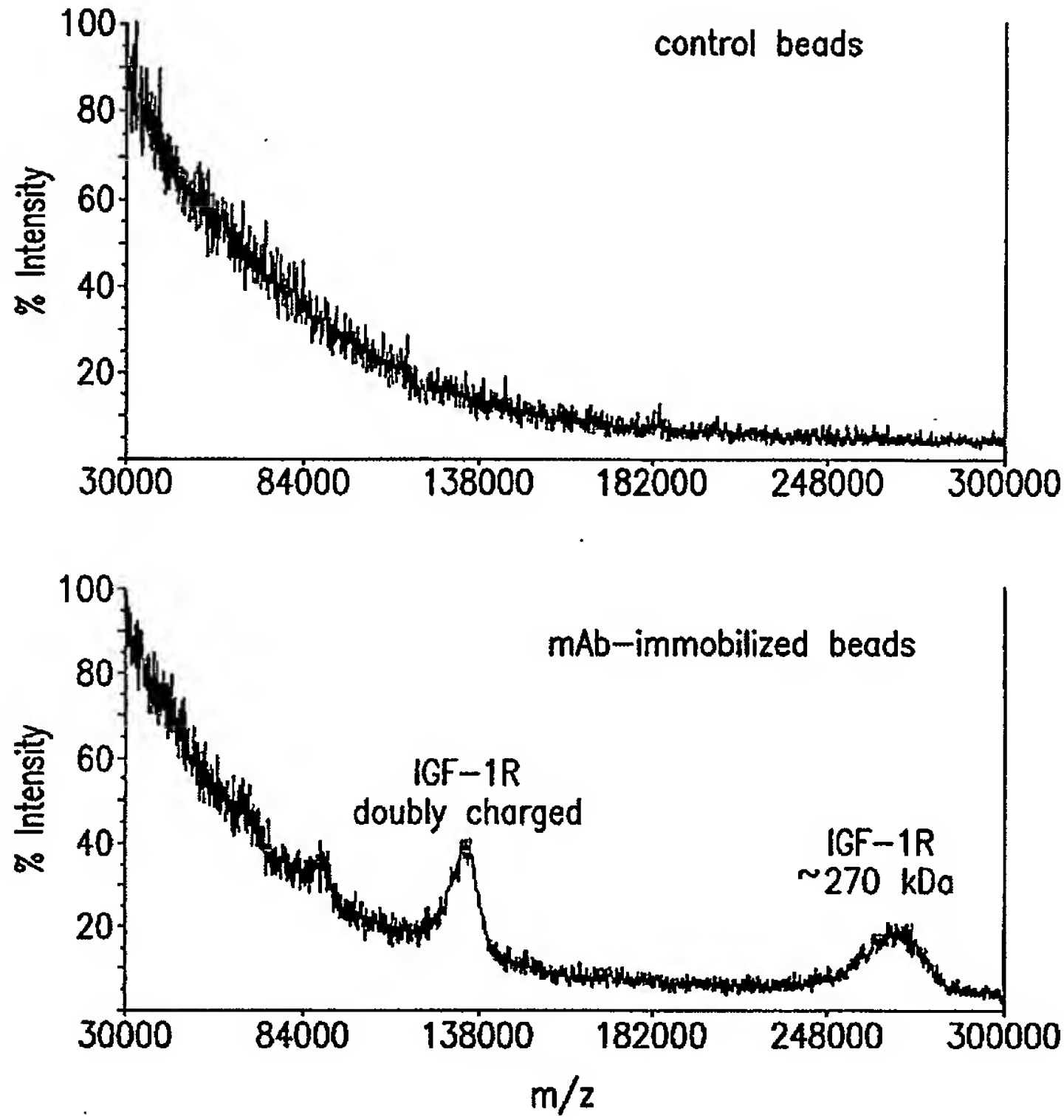

control beads
% Intensity
100
80
60
40
20
30000
84000
138000
182000
248000
300000
mAb–immobilized beads
IGF–1R
doubly charged
IGF–1R
~270 kDa
m/z

FIG. 1

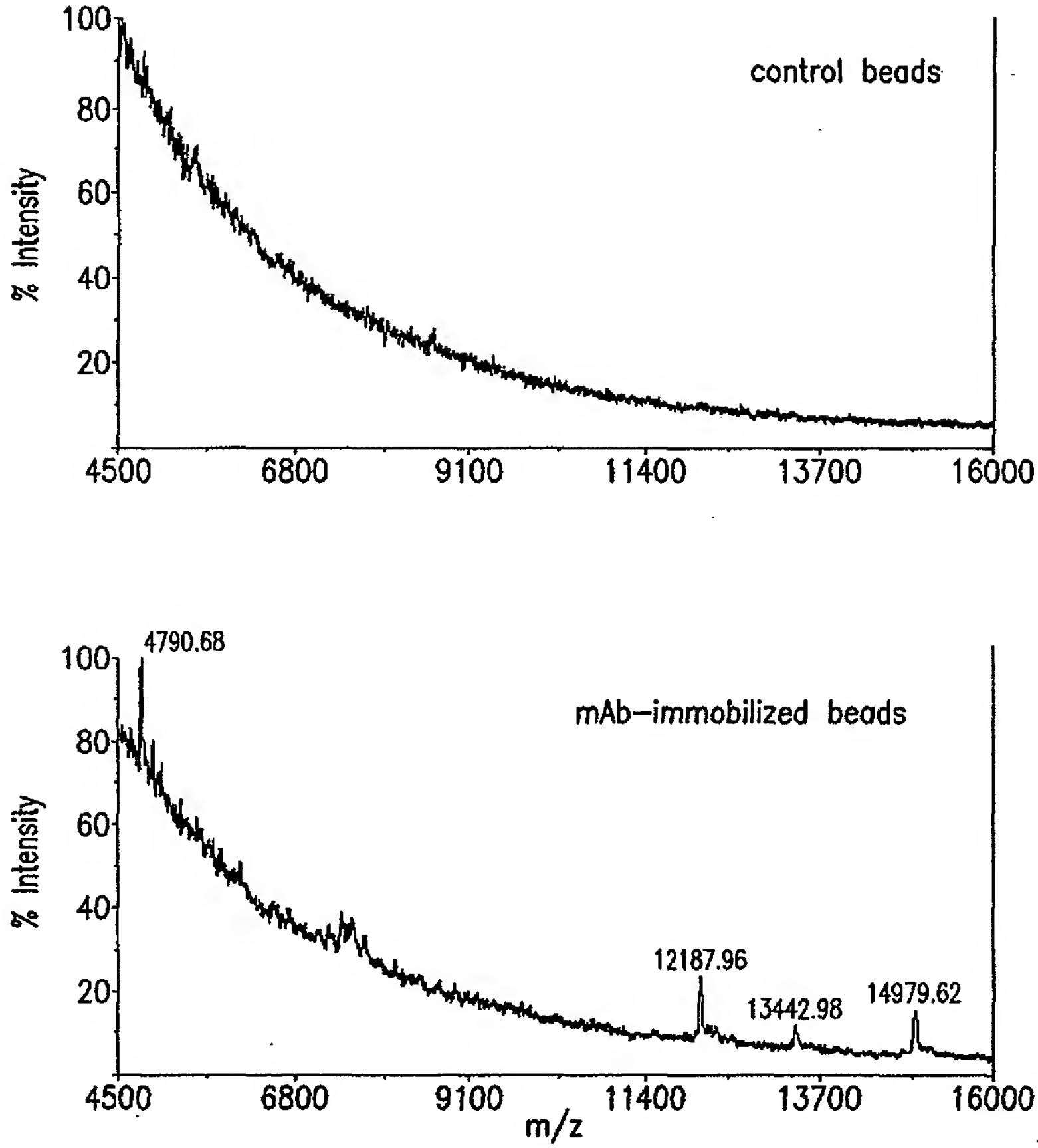

control beads
% Intensity
100
80
60
40
20
4500
6800
9100
11400
13700
16000
mAb–immobilized beads
4790.68
12187.96
13442.98
14979.62
m/z

FIG.2

EICGPGIDIRNDYQQLKRLENCTVIEGYLHILLISKAEDYRSYRFPKLTVITEYLLLFRVAGLESLG
DLFPNLTVIRGWKLFYNYALVIFEMTNLKDIGLYNLRNITRGAIRIEKNADLCYLSTVDWSLILDA
VSNNYIVGNKPPKECGDLCPGTMEEKPMCEKTTINNEYNYRCWTTNRCQKMCPSTCGKRACTE
NNECCHPECLGSCSAPDNDTACVACRHYYYAGVCVPACPPNTYRFEGWRCVDRDFCANILSAE
SSDSEGFVIHDGECMQECPSGFIRNGSQSMYCIPCEGPCPKVCEEEKKTKTIDSVTSAQMLQGCTI
FKGNLLINIRRGNNIASELENFMGLIEVVTGYVKIRHSHALVSLSFLKNLRLILGEEQLEGNYSFYV
LDNQNLQQLWDWDHRNLTIKAGKMYFAFNPKLCVSEIYRMEEVTGTKGRQSKGDINTRNNGER
ASCESDVLHFTSTTTSKNRIIITWHRYRPPDYRDLISFTVYYKEAPFKNVTEYDGQDACGSNSWN
MVDVDLPPNKDVEPGILLHGLKPWTQYAVYVKAVTLTMVENDHIRGAKSEILYIRTNASVPSIPL
DVLSASNSSSQLIVKWNPPSLPNGNLSYYIVRWQRQPQDGYLYRHNYCSKDKIPIRKYADGTIDI
EEVTENPKTEVCGGEKGPCCACPKTEAEKQAEKEEAEYRKVFENFLHNSIFVPRPERKRR

FIG.3

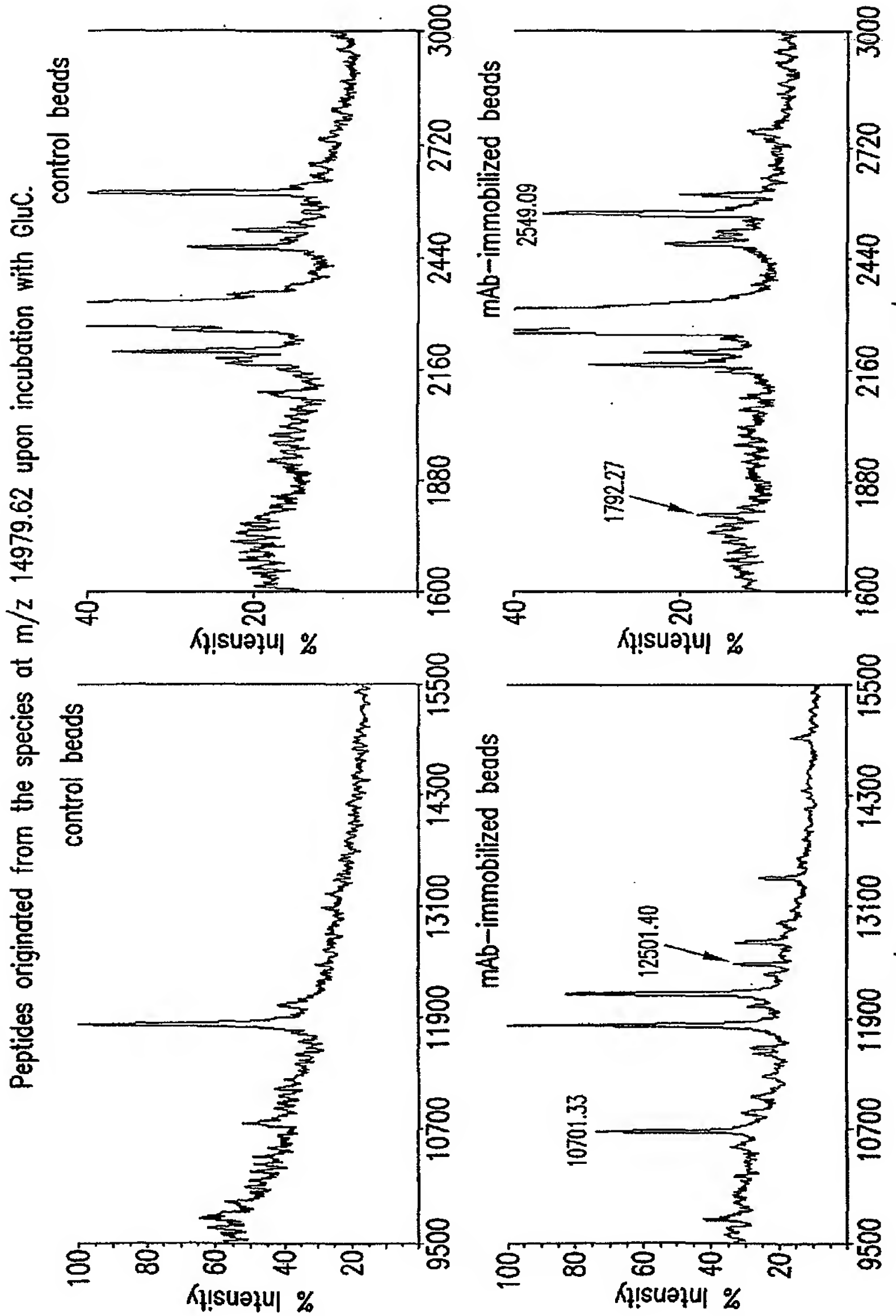

Peptides originated from the species at m/z 14979.62 upon incubation with GluC.
control beads
mAb-immobilized beads
2549.09
1792.27
12501.40
10701.33
% Intensity
m/z
100
80
60
40
20
9500
10700
11900
13100
14300
15500
1600
1880
2160
2440
2720
3000

FIG.4

EICGPGIDIR	NDYQQLKRLE	NCTVIEGYLH	ILLISKAEDY	RSYRFPKLTV
ITEYLFRV	AGLESLGDLF	PNLTVIRGWK	LFYNYALVIF	EMTNLKDIGL
YNLRNITRGA	IRIEKNADLC	YLSTVDWSLI	LDAVSNNYIV	GNKPPKECGD
LCPGTMEEKP	MCEKTTINNE	YNYRCWTTNR	CQKMCPSTCG	KRACTENNEC
CHPECLGSCS	APDNDTACVA	CRHYYYAGVC	VPACPPNTYR	FEGWRCVDRD
ECANILSAES	SDSEGFVIHD	GECMQECPSG	FIRNGSQSMY	CIPCEGPCPK
VCEEEKKTKT	IDSVTSAQML	QGCTIFKGNL	LINIRRGNNI	ASELENFMGL
IEVVTGYVKI	RHSHALVSLS	FLKNLRLILG	EEQLEGNYSF	YVLDNQNLQQ
LWDWDHRNLT	IKAGKMYFAF	NPKLCVSEIY	RMEEVTGTKG	RQSKGDINTR
NNGERASCES	DVLHFTSTTT	SKNRIIITWH	RYRPPDYRDL	ISFTVYYKEA
PFKNVTEYDG	QDACGSNSWN	MVDVDLPPNK	DVEPGILLHG	LKPWTQYAVY
VKAVTLTMVE	NDHIRGAKSE	ILYIRTNASV	PSIPLDVLSA	SNSSSQLIVK
WNPPSLPNGN	LSYYIVRWQR	QPQDGYLYRH	NYCSKDKIPI	RKYADGTIDI
EEVTENPKTE	VCGGEKGPCC	ACPKTEAEKQ	AEKEEAEYRK	VFENFLHNSI
EVPRPERKRR				

F50035 epitope

IGF-1-binding residues

IGF-1R α-chain

(IGF-1 binding residues are represented as shaded while the boxed region(s) comprise the epitope, bound by the reference antibody)

FIG.5

```
hIGF-1R(27-41) GYLHILLISKAEDYRSYR
mIGF-1R(27-41) GFLHILLISKAEDYRSYR
rIGF-1R(27-41) GFLHILLISKAEDYRSYR
hIGF-1R(688-707) YRKVFENFLHNSIFVPRPER
mIGF-1R(688-707) YRKVFENFLHNSIFVPRPER
rIGF-1R(688-707) YRKVFENFLHNSIFVPRPER
```

FIG.6

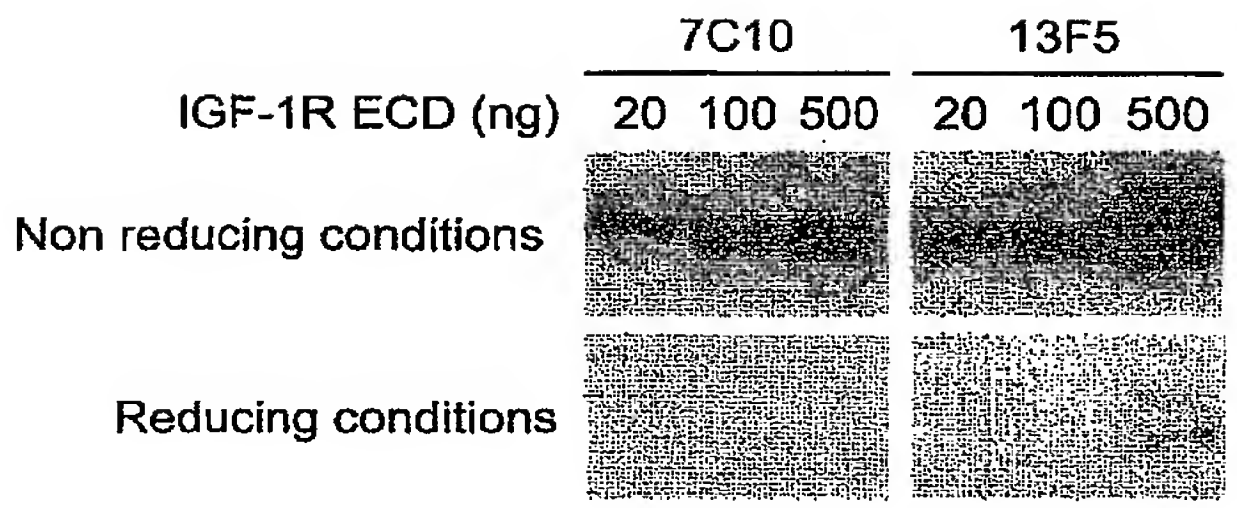
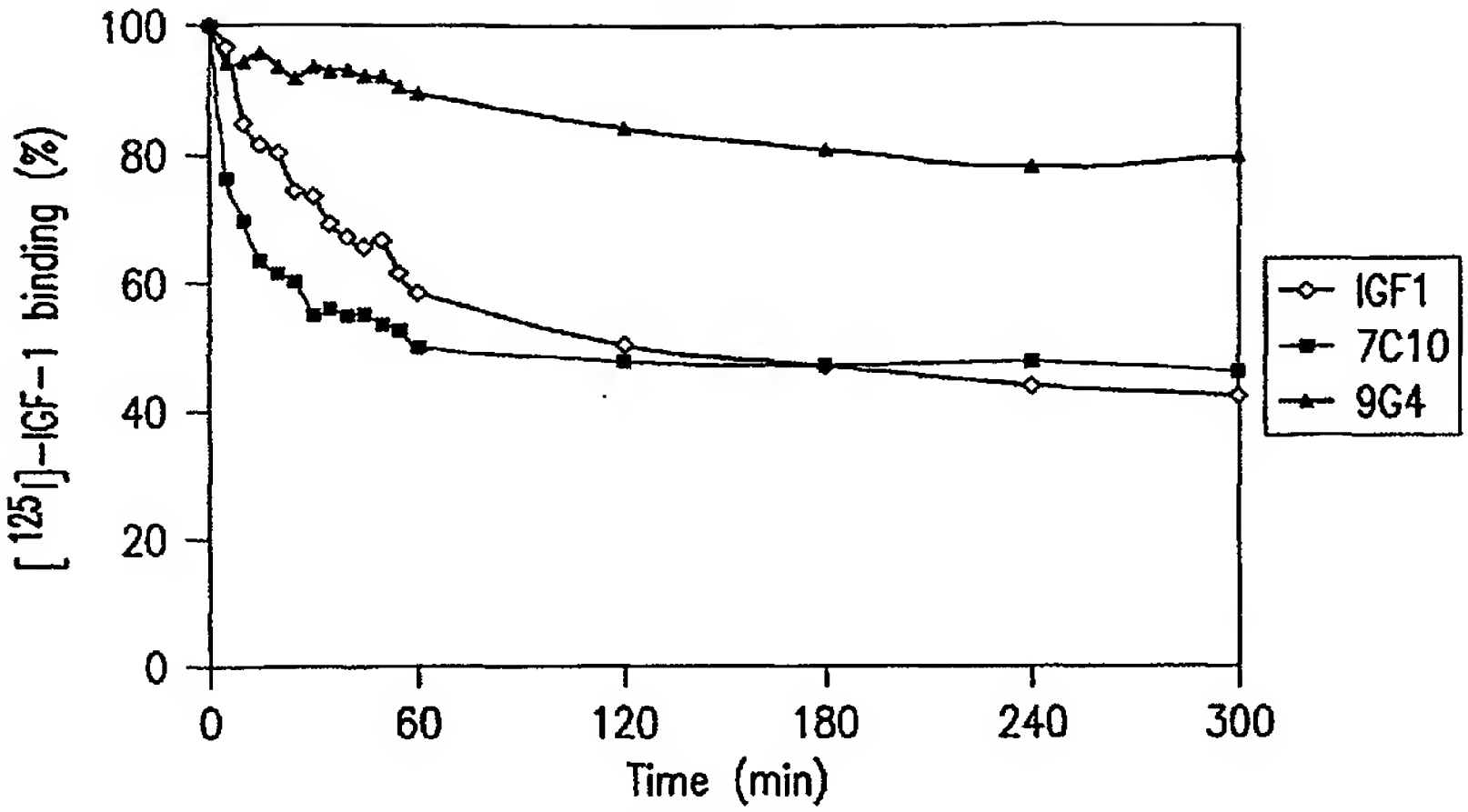
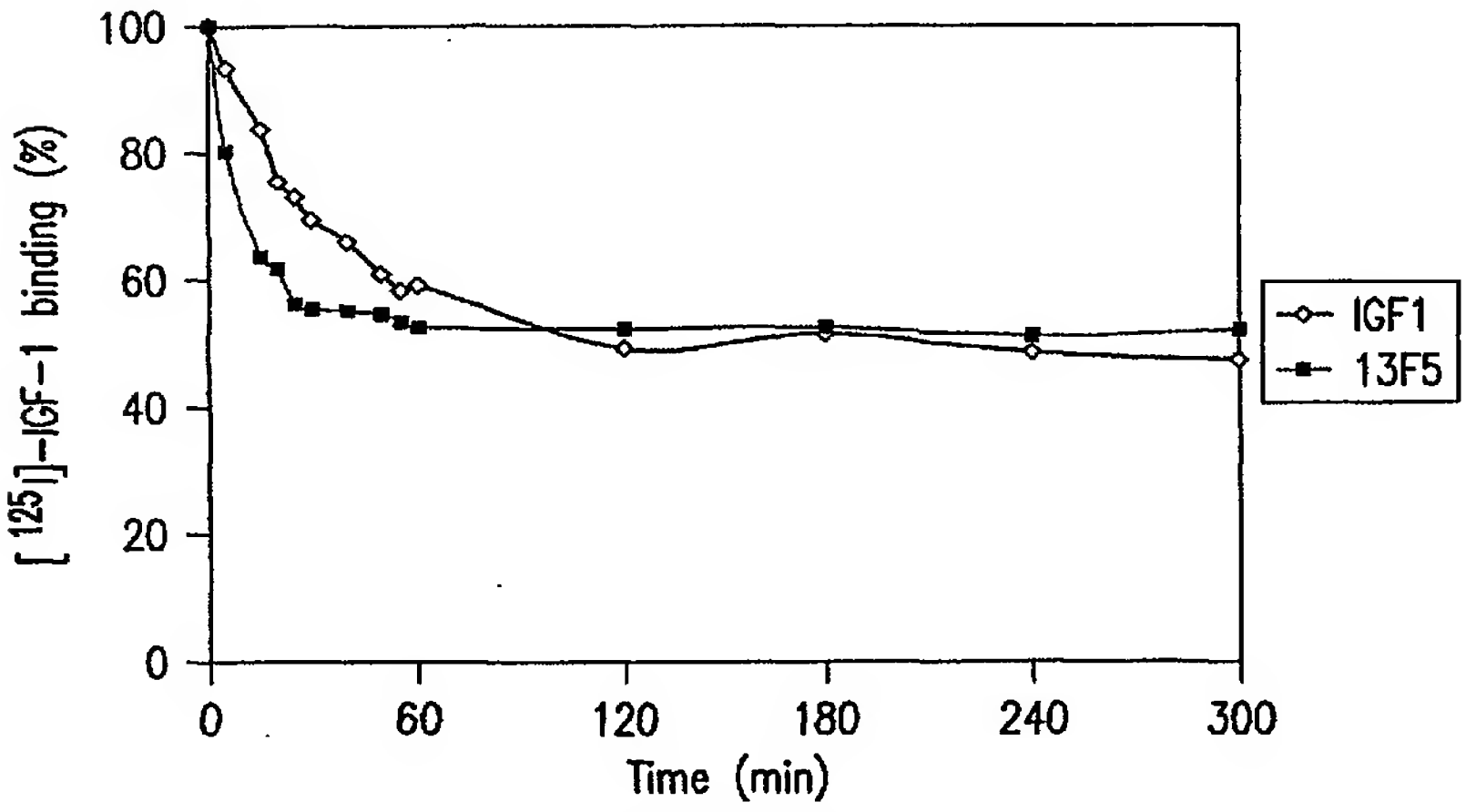

7C10
13F5
IGF-1R ECD (ng)
20 100 500
20 100 500
Non reducing conditions
Reducing conditions

FIG.7



Dissociation of [^{125}I]-IGF-1/IGF-1R complexes by the monoclonal antibodies 7C10 (A) and 13F5 (B).

FIG.8A



Dissociation of [^{125}I]-IGF-1/IGF-1R complexes by the monoclonal antibodies 7C10 (A) and 13F5 (B).

FIG.8B

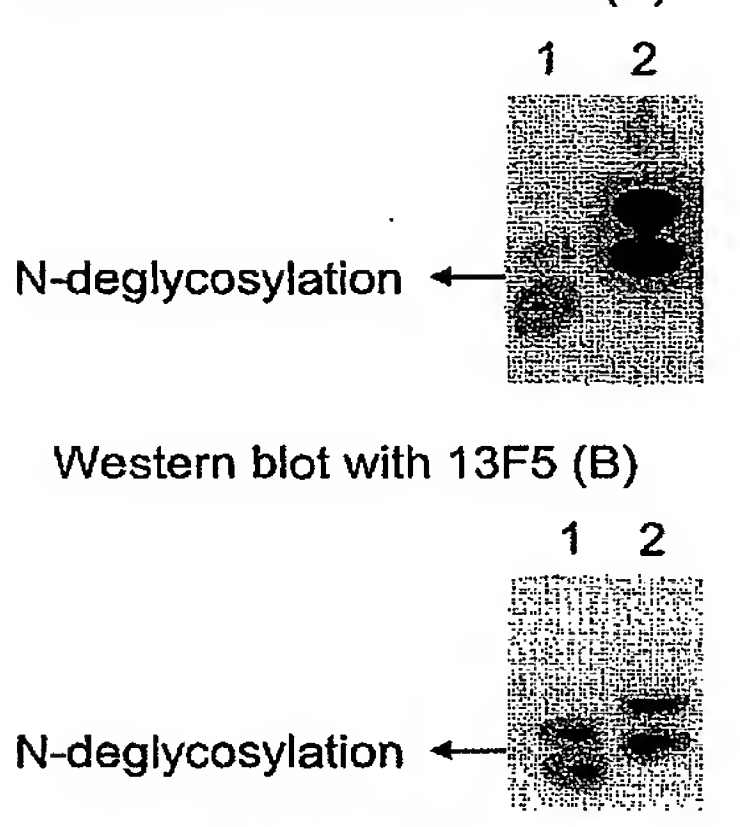

Western blot with A2CHM (A)
1 2
N-deglycosylation
Western blot with 13F5 (B)
1 2
N-deglycosylation

FIG.9

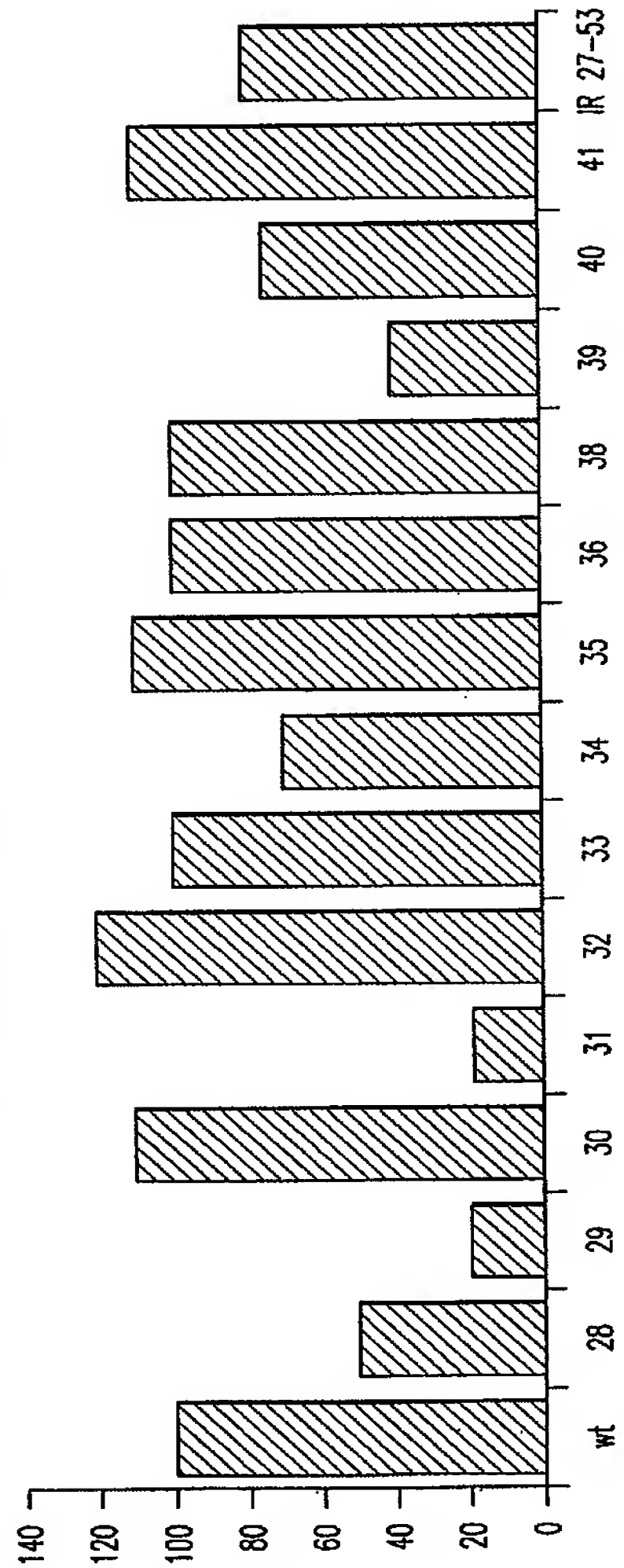
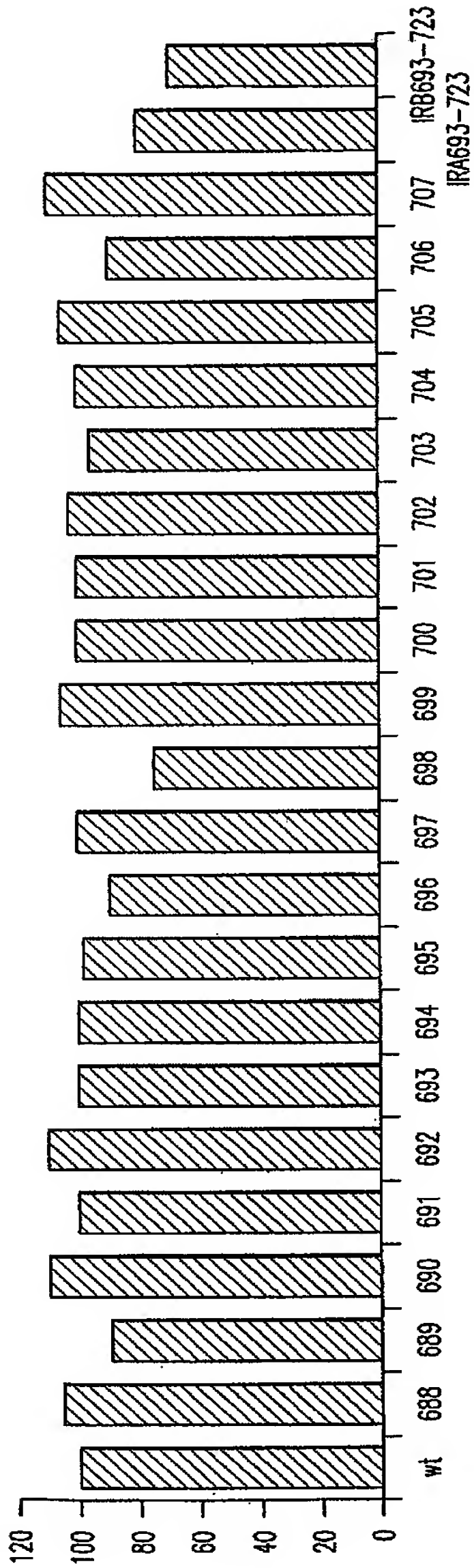

Ala scanning mutants in the N-terminus region
140
120
100
80
60
40
20
0
wt
28
29
30
31
32
33
34
35
36
38
39
40
41
IR 27-53

FIG.10A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IRB693-723
IRA693-723
707
706
705
704
703
702
701
700
699
698
697
696
695
694
693
692
691
690
689
688
wt
120
100
80
60
40
20
0

FIG.10B

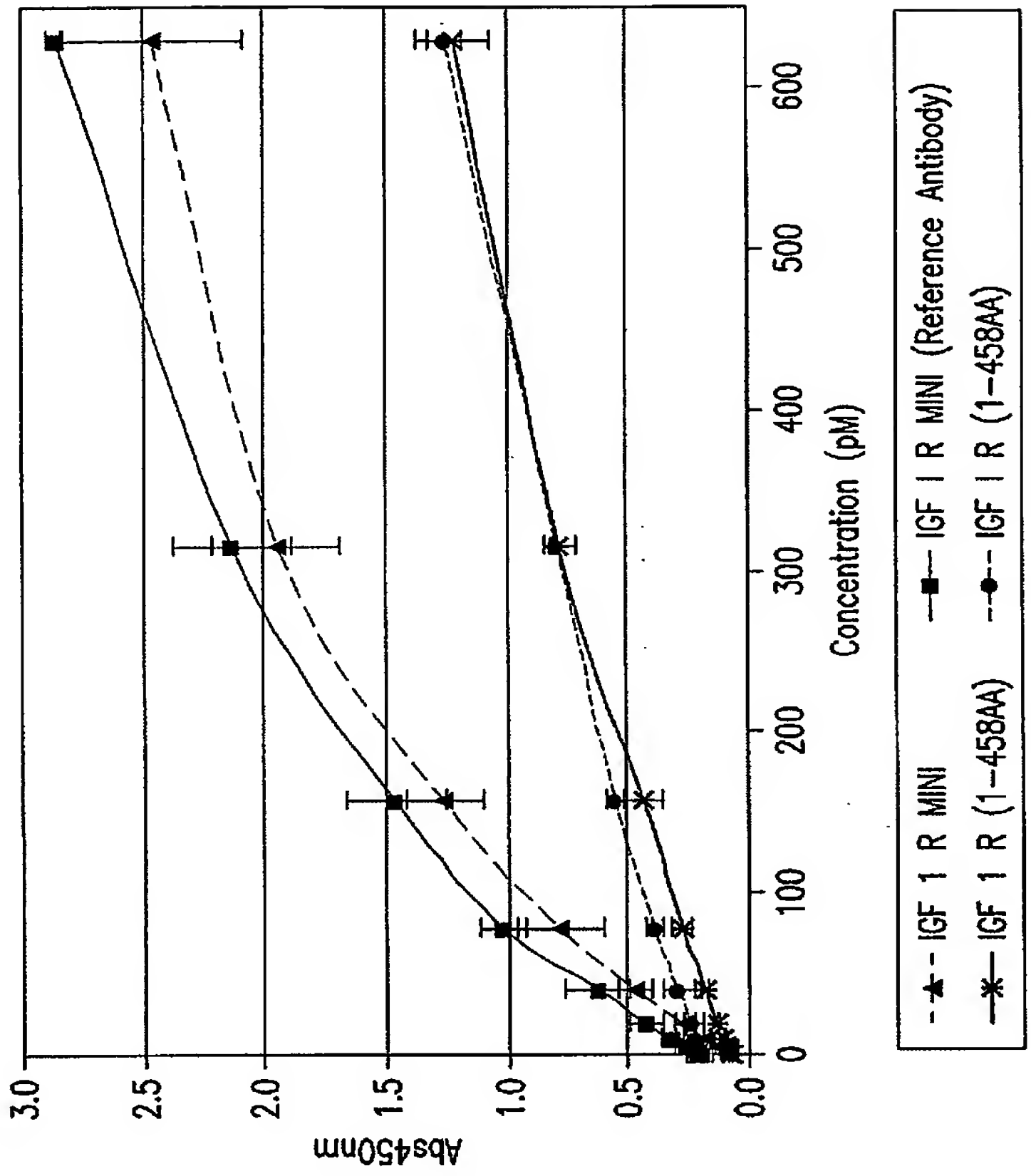

Abs450nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
100
200
300
400
500
600
Concentration (pM)
IGF 1 R MINI
IGF 1 R (1–458AA)
IGF I R MINI (Reference Antibody)
IGF I R (1–458AA)

FIG. 11

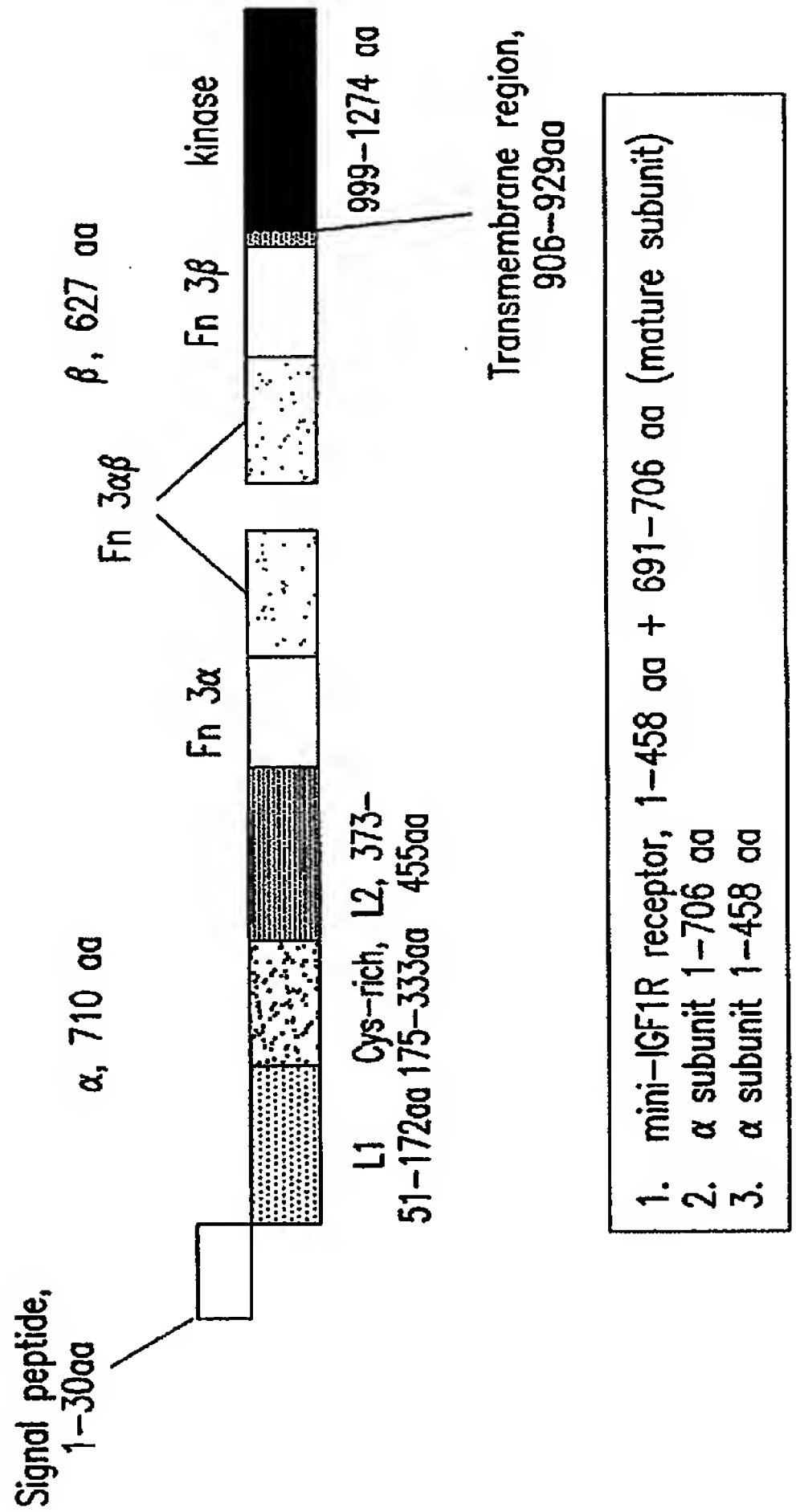

Constructs of IGF1R
α, 710 aa
β, 627 aa
Fn 3αβ
Fn 3α
Fn 3β
kinase
999–1274 aa
Transmembrane region,
906–929aa
L1 Cys-rich, L2, 373-
51–172aa 175–333aa 455aa
Signal peptide,
1–30aa
1. mini–IGF1R receptor, 1–458 aa + 691–706 aa (mature subunit)
2. α subunit 1–706 aa
3. α subunit 1–458 aa

FIG.12A

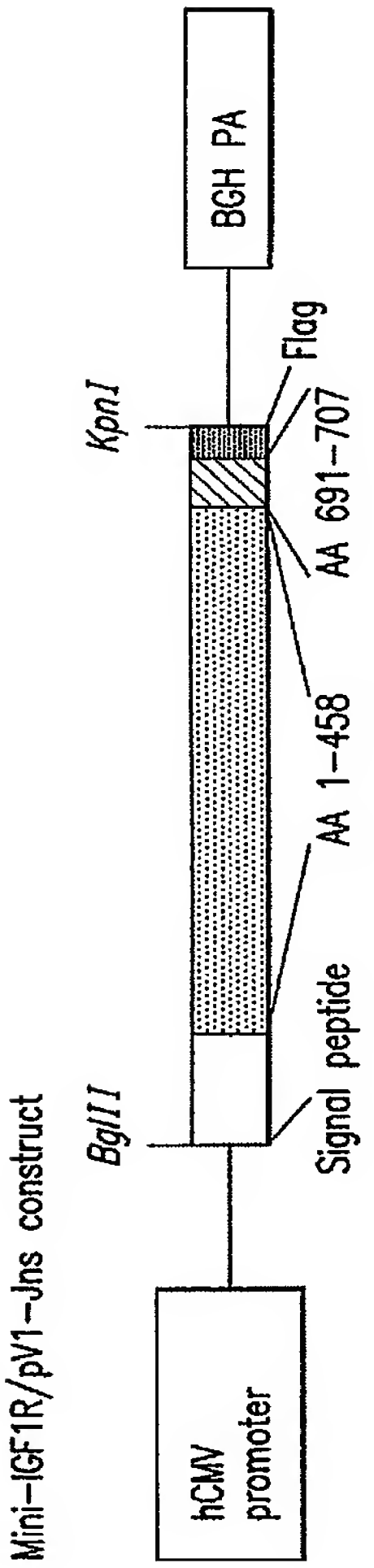


FIG.12B

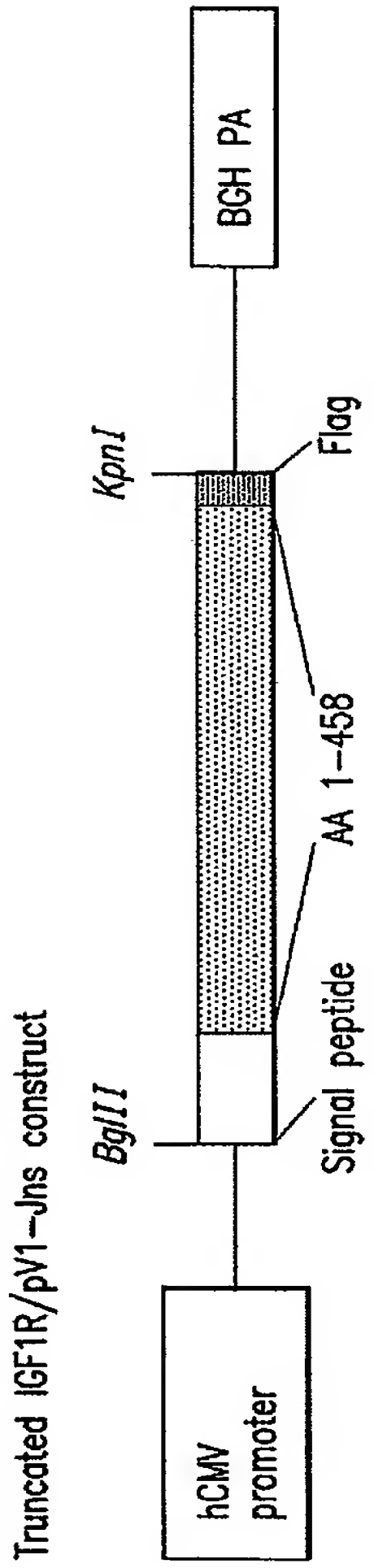


FIG.12C

Forward primer:
5'– ACTCAT*AGATCT*AGTATGGCGTCTGGCTCCGGAG–3'

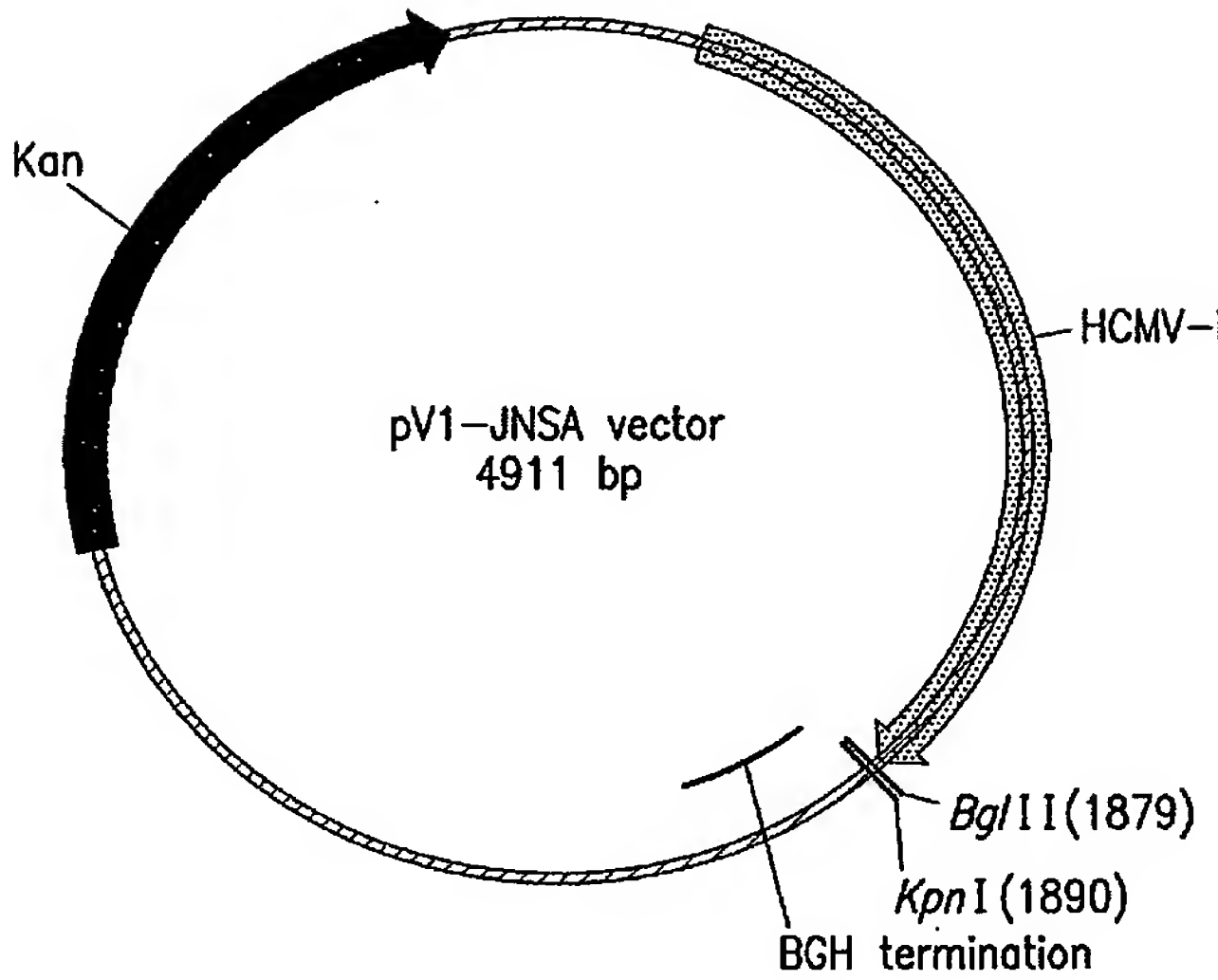
FIG.12D

Reverse primer for mini–IGF1R:
5'– ATATCT*GGTACC*AACCTACTTGTCATCGTCGTCCTTGTAATCTTCAGGTCTGGGCAC
GAAGATGGAGTTGTGCAGGAAATTCTCAAAGACACAGGAGGCTCTCTCCCCGTTG–3'

FIG.12E

Reverse primer for truncated IGF1R:
5'– ATATCT*GGTACC*AACCTACTTGTCATCGTCGTCCTTGTAATCACAGGAGGCTCTCTCCCC
GTTG–3'

FIG.12F


Kan
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pV1-JNSA vector
4911 bp
BglII(1879)
KpnI(1890)
BGH termination

FIG.12G

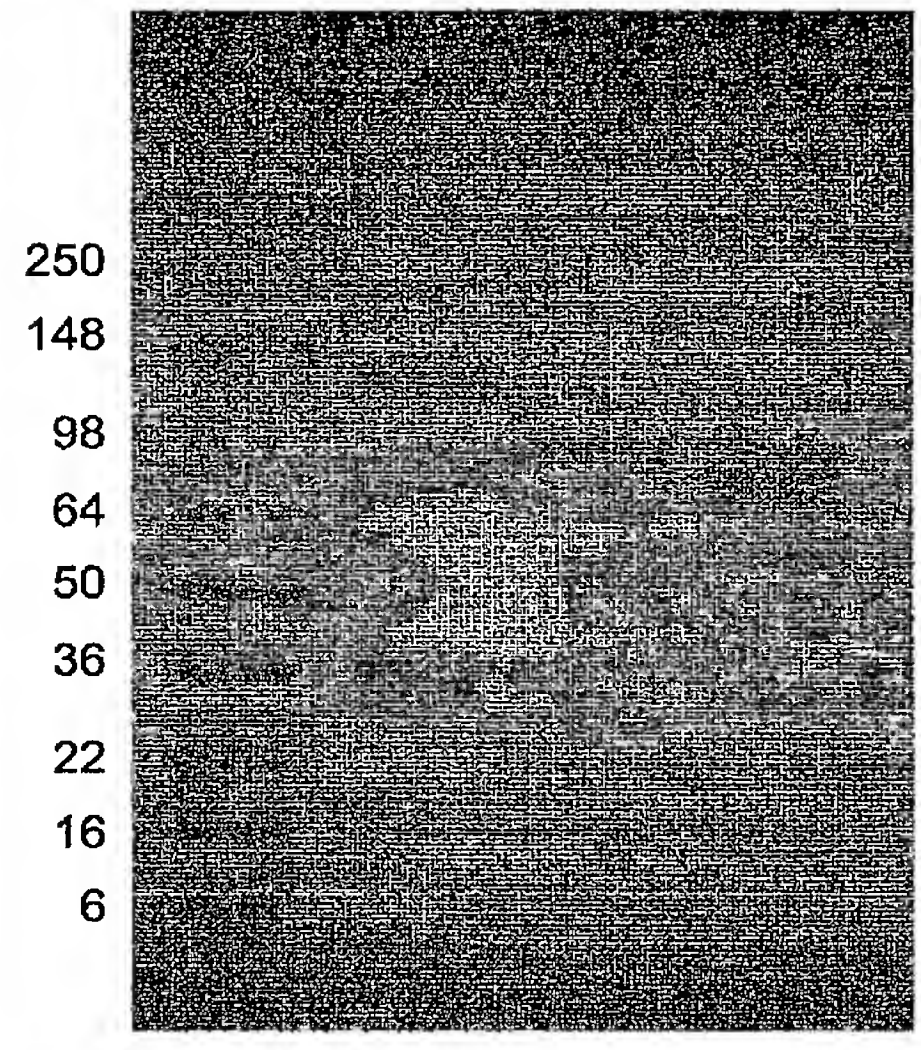

FIG.12H

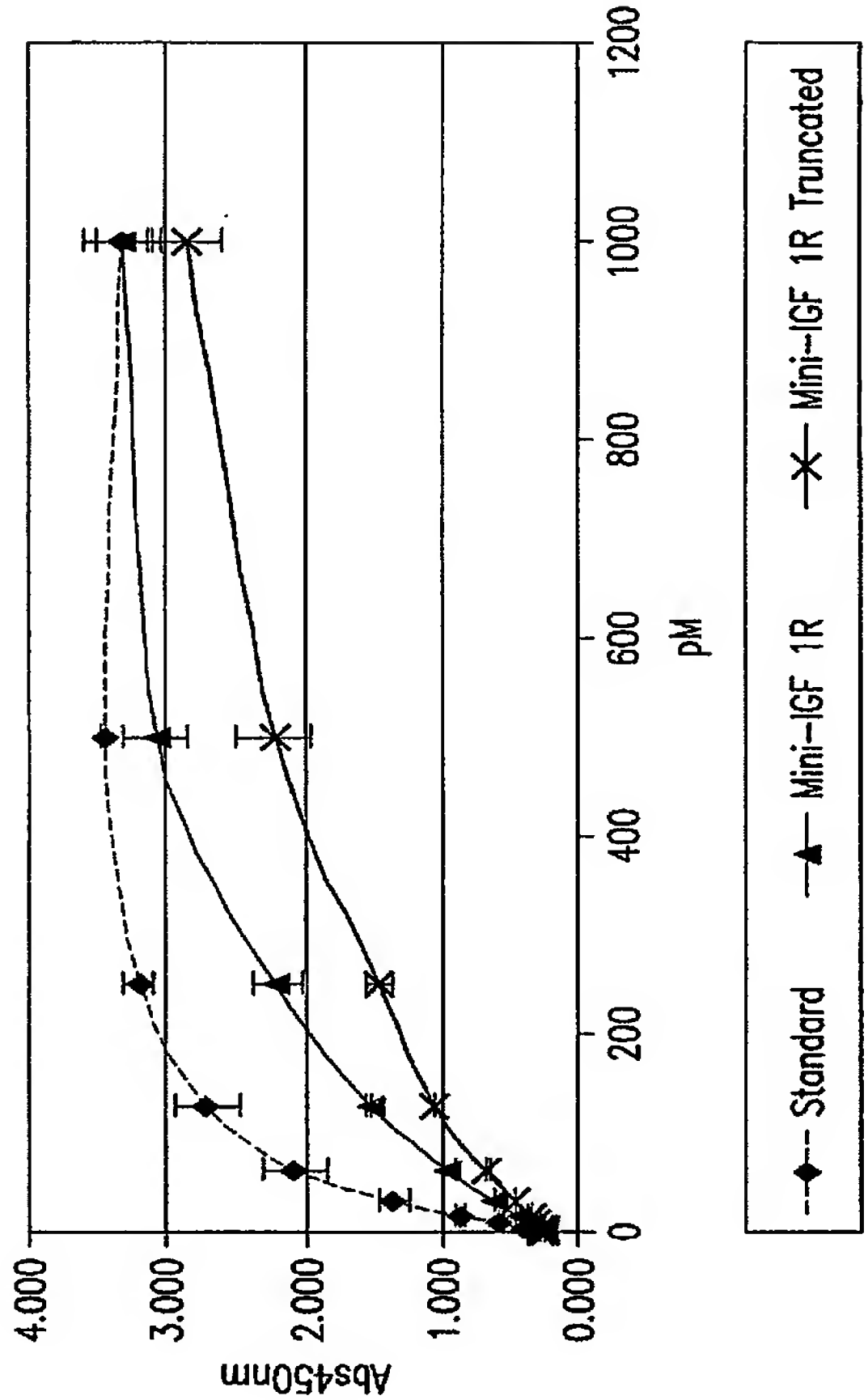
Abs450nm
4.000
3.000
2.000
1.000
0.000
0
200
400
600
800
1000
1200
pM
Standard
Mini-IGF 1R
Mini-IGF 1R Truncated

FIG.12I

Schematic representation of the biosensor capturing assay

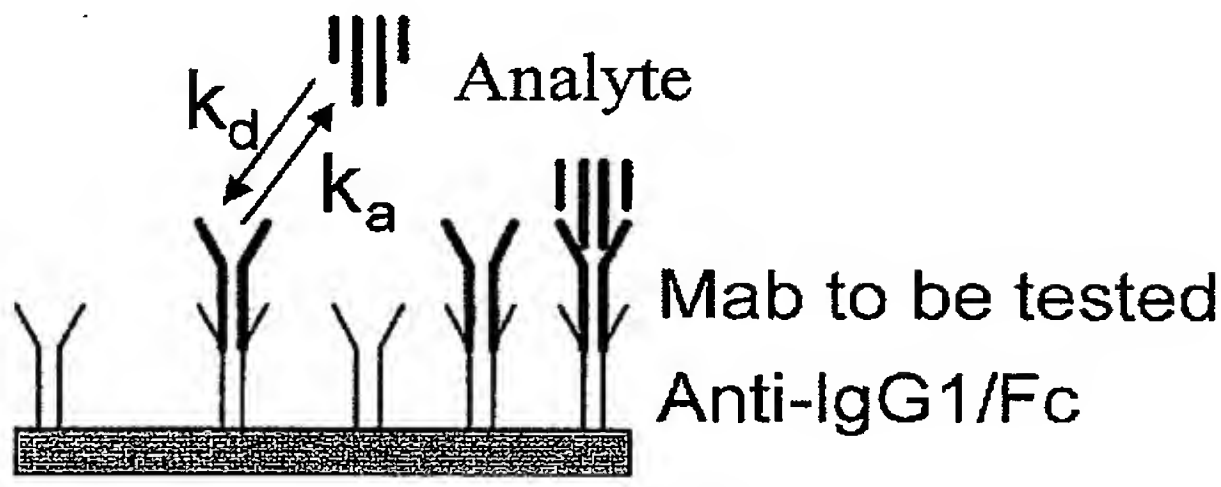

FIG. 13

Sensorgram of the association and dissociation phase of reference antibody (F50035 mAb)/analyte complexes for five different concentrations of the respective analytes

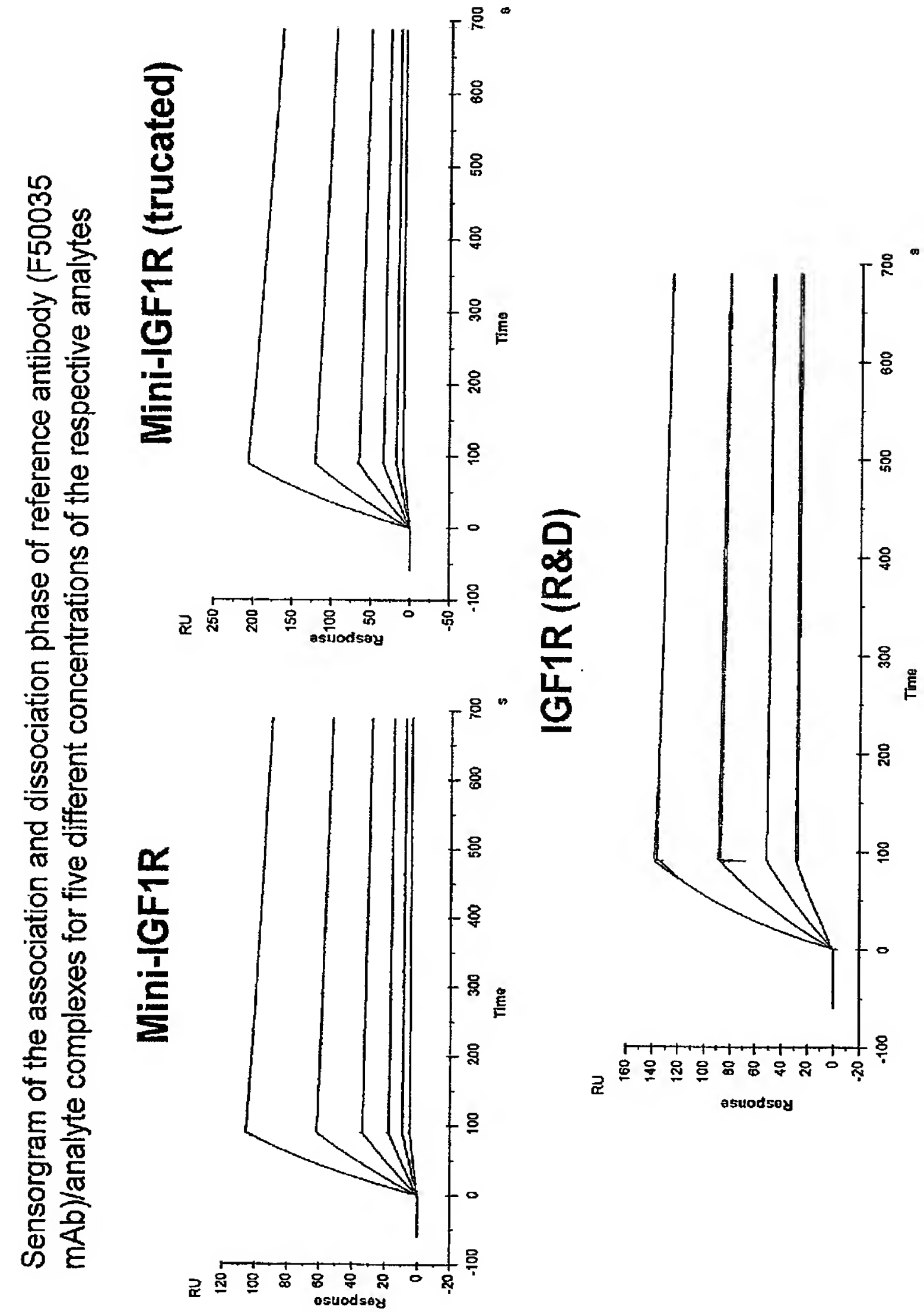

FIG. 14

ITR0091Y.TXT

SEQUENCE LISTING

<110> Merck & Co., Inc.
Haeuw, Jean-Francois
Klinguer-Hamour, Christine
Pessi, Antonello
Talamo, Fabio

<120> ANTIBODIES THAT BIND TO AN EPITOPE ON
INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR AND USES THEREOF

<130> ITR0091Y - PCT

<140> To Be Assigned
<141>

<150> US 60/694,802
<151> 2005-06-27

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<151> 2005-07-18

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Arg Pro Glu Arg
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Leu Ile Ser Lys Ala Glu Asp Tyr Arg Ser Tyr Arg Phe Pro Lys Leu
35 40 45
Thr Val Ile Thr Glu Tyr Leu Leu Leu Phe Arg Val Ala Gly Leu Glu
50 55 60
Ser Leu Gly Asp Leu Phe Pro Asn Leu Thr Val Ile Arg Gly Trp Lys
65 70 75 80
Leu Phe Tyr Asn Tyr Ala Leu Val Ile Phe Glu Met Thr Asn Leu Lys
85 90 95

```
Asp Ile Gly Leu Tyr Asn Leu Arg Asn Ile Thr Arg Gly Ala Ile Arg
                100                 105                 110
Ile Glu Lys Asn Ala Asp Leu Cys Tyr Leu Ser Thr Val Asp Trp Ser
            115                 120                 125
Leu Ile Leu Asp Ala Val Ser Asn Asn Tyr Ile Val Gly Asn Lys Pro
    130                 135                 140
Pro Lys Glu Cys Gly Asp Leu Cys Pro Gly Thr Met Glu Glu Lys Pro
145                 150                 155                 160
Met Cys Glu Lys Thr Thr Ile Asn Asn Glu Tyr Asn Tyr Arg Cys Trp
                165                 170                 175
Thr Thr Asn Arg Cys Gln Lys Met Cys Pro Ser Thr Cys Gly Lys Arg
            180                 185                 190
Ala Cys Thr Glu Asn Asn Glu Cys Cys His Pro Glu Cys Leu Gly Ser
        195                 200                 205
Cys Ser Ala Pro Asp Asn Asp Thr Ala Cys Val Ala Cys Arg His Tyr
    210                 215                 220
Tyr Tyr Ala Gly Val Cys Val Pro Ala Cys Pro Pro Asn Thr Tyr Arg
225                 230                 235                 240
Phe Glu Gly Trp Arg Cys Val Asp Arg Asp Phe Cys Ala Asn Ile Leu
                245                 250                 255
Ser Ala Glu Ser Ser Asp Ser Glu Gly Phe Val Ile His Asp Gly Glu
            260                 265                 270
Cys Met Gln Glu Cys Pro Ser Gly Phe Ile Arg Asn Gly Ser Gln Ser
        275                 280                 285
Met Tyr Cys Ile Pro Cys Glu Gly Pro Cys Pro Lys Val Cys Glu Glu
    290                 295                 300
Glu Lys Lys Thr Lys Thr Ile Asp Ser Val Thr Ser Ala Gln Met Leu
305                 310                 315                 320
Gln Gly Cys Thr Ile Phe Lys Gly Asn Leu Leu Ile Asn Ile Arg Arg
                325                 330                 335
Gly Asn Asn Ile Ala Ser Glu Leu Glu Asn Phe Met Gly Leu Ile Glu
            340                 345                 350
Val Val Thr Gly Tyr Val Lys Ile Arg His Ser His Ala Leu Val Ser
        355                 360                 365
Leu Ser Phe Leu Lys Asn Leu Arg Leu Ile Leu Gly Glu Glu Gln Leu
    370                 375                 380
Glu Gly Asn Tyr Ser Phe Tyr Val Leu Asp Asn Gln Asn Leu Gln Gln
385                 390                 395                 400
Leu Trp Asp Trp Asp His Arg Asn Leu Thr Ile Lys Ala Gly Lys Met
                405                 410                 415
Tyr Phe Ala Phe Asn Pro Lys Leu Cys Val Ser Glu Ile Tyr Arg Met
            420                 425                 430
Glu Glu Val Thr Gly Thr Lys Gly Arg Gln Ser Lys Gly Asp Ile Asn
        435                 440                 445
Thr Arg Asn Asn Gly Glu Arg Ala Ser Cys Glu Ser Asp Val Leu His
    450                 455                 460
Phe Thr Ser Thr Thr Thr Ser Lys Asn Arg Ile Ile Ile Thr Trp His
465                 470                 475                 480
Arg Tyr Arg Pro Pro Asp Tyr Arg Asp Leu Ile Ser Phe Thr Val Tyr
                485                 490                 495
Tyr Lys Glu Ala Pro Phe Lys Asn Val Thr Glu Tyr Asp Gly Gln Asp
            500                 505                 510
Ala Cys Gly Ser Asn Ser Trp Asn Met Val Asp Val Asp Leu Pro Pro
        515                 520                 525
Asn Lys Asp Val Glu Pro Gly Ile Leu Leu His Gly Leu Lys Pro Trp
    530                 535                 540
Thr Gln Tyr Ala Val Tyr Val Lys Ala Val Thr Leu Thr Met Val Glu
545                 550                 555                 560
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gttg 64

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference ITR0091Y	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/EP2006/006221	International filing date *(day/month/year)* 26/06/2006	(Earliest) Priority Date *(day/month/year)* 27/06/2005
Applicant ISTITUTO DI RICERCHE DI BIOLOGIA MOLECOLARE ...		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [X] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [X] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. 3
      [X] as suggested by the applicant
      [ ] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [ ] none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2006/006221

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] on paper

   [X] in electronic form

   c. time of filing/furnishing

   [X] contained in the international application as filed

   [X] filed together with the international application in electronic form

   [ ] furnished subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/006221

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28 A61K39/395 C12N5/20 C12N15/63 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/059951 A (PIERRE FABRE MEDICAMENT; GOETSCH, LILIANE; CORVAIA, NATHALIE; LEGER, O) 24 July 2003 (2003-07-24) cited in the application the whole document claims 1-54	1-19,24, 26,27
Y		20-23
Y	WO 02/053596 A (PFIZER INC; ABGENIX, INC; COHEN, BRUCE, D; BEEBE, JEAN; MILLER, PENELO) 11 July 2002 (2002-07-11) page 8; claims 1-33; examples 1-18	20-23

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 October 2006	19/10/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Bernhardt, Wiebke

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/006221

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ULLRICH A ET AL: "INSULIN-LIKE GROWTH FACTOR I RECEPTOR PRIMARY STRUCTURE: COMPARISON WITH INSULIN RECEPTOR SUGGESTS STRUCTURAL DETERMINANTS THAT DEFINE FUNCTIONAL SPECIFICITY" EMBO JOURNAL, IRL PRESS, EYNSHAM, GB, vol. 5, no. 10, 1986, pages 2503-2512, XP000650155 ISSN: 0261-4189 cited in the application the whole document figures 2,3	3
P,X	WO 2005/058967 A (PIERRE FABRE MEDICAMENT; GOETSCH, LILIANE; CORVAIA, NATHALIE; DUFLOS,) 30 June 2005 (2005-06-30) cited in the application the whole document	1-19,24, 26,27
A	LI S-L ET AL: "SINGLE-CHAIN ANTIBODIES AGAINST HUMAN INSULIN-LIKE GROWTH FACTOR I RECEPTOR: EXPRESSION, PURIFICATION AND EFFECT ON TUMOR GROWTH" CANCER IMMUNOLOGY AND IMMUNOTHERAPY, BERLIN, DE, vol. 49, no. 4/5, July 2000 (2000-07), pages 243-252, XP001113064 ISSN: 0340-7004 the whole document	1-28
A	WO 2005/005635 A (F. HOFFMANN-LA ROCHE AG; GRAUS, YVO; KOPETZKI, ERHARD; KUENKELE, KLAUS) 20 January 2005 (2005-01-20) pages 3-7; claims 1-19	1-28
A	WO 03/106621 A (IMMUNOGEN, INC) 24 December 2003 (2003-12-24) pages 7-10; claims 1-61	1-28

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2006/006221

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 12-14 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2006/006221

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 03059951 A	24-07-2003	AU	2003216748 A1	30-07-2003
		BR	0306973 A	09-11-2004
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		CN	1823163 A	23-08-2006
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		EP	1532174 A2	25-05-2005
		JP	2005533493 T	10-11-2005
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